FAT METABOLISM

A Symposium on the Clinical and Biochemical Aspects of Fat Utilization in Health and Disease

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A Symposium on Fat Metabolism

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A Symposium on the Clinical and Biochemical Aspects of Fat Utilization in Health and Disease

Edited by

VICTOR A. NAJJAR

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A Symposium on Fat Metabolism

1. Clinical and Biochemical Features of Fat Metabolism: An Introduction

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THE PROBLEM OF OBESITY and fat metabolism has been the concern of the medical clinician and the medical biochemist for quite some time. Interest in fat metabolism until a few years ago was merely an offspring of a wider interest focused on carbohydrate metabolism. This perhaps is the natural course of events, inasmuch as carbohydrates generally contribute over 50 per cent of the caloric intake and obesity is therefore due largely to the conversion and deposition of excess carbohydrates in the fat stores. Moreover, diabetes, the disease par excellence of sugar metabolism, is often associated with increased output of ketone bodies which are products of incomplete fatty acid oxidation. Recently, the successful search for the 2-carbon fragment at the last stages of glycolysis, as elucidated by Lipmann, has renewed interest in fat metabolism by opening the way for an understanding of fat synthesis and breakdown at the enzyme level.

Obesity as a clinical entity is ill-defined and obscure. This is mainly due to the lack of understanding of the many factors that give rise to the obese individual. There are instances where an endocrine disturbance can cause excessive deposition of fat such as one encounters in cases of adrenocortical hyperfunction due to adrenocortical tumors or hyperplasia as exemplified by Cushing's syndrome. Cortisone remains the only physiological compound that can cause obesity when administered in excess of body needs. This type of obesity is indistinguishable from that of Cushing's syndrome. Other than these instances, the causes of obesity abide, for the present at least, in the realm of uncertainty. There are *psychic, environmental, traumatic* and *genetic* factors that may be involved in its etiology. These are necessarily complex causes and require the assistance of factors more intimately concerned with metabolism and the balance between caloric intake and expenditure of energy. Thus a child who is psychologically disturbed and finds refuge in overeating may not gain weight if his redoubt includes overactivity. Similarly, another child with the same disturbance can materially augment his adipose tissue if his refuge is limited to inactivity and sluggishness without affecting his food intake. Postencephalitic obesity is in many cases due to restriction of activity with no concomitant diminution of caloric intake. While there is little doubt that some forms of obesity run in families, it is nevertheless difficult in such instances to separate the genetic and environmetal factors that might be involved. Hereditary types of obesity, however, have been described and studied in animals.

Whether food is conserved in fat depots or fully metabolized depends on the caloric intake as well as on the demand for metabolism. Food intake is governed by the appetite as well as by a host of undefined conditions, physiological, psychological, and pathological. Freed from these shackles, food intake may be regulated by a " glucostatic mechanism." " Gluco-receptors " present in the hypothalamus are sensitive to arteriovenous differences in glucose level. When the difference between the arterial and venous glucose levels approaches zero, contractions of the stomach are stimulated and hunger sensations are felt. A difference of over 10 mg. per cent is not associated with hunger contractions. Obese individuals in general show a magnitude of arteriovenous difference that is generally greater than normal.

The ingested fat is hydrolyzed by the lipases of the gastro-intestinal tract before absorption by the mucosa. A reconstitution of fat by esterification of the fatty acids occurs in the intestinal mucosa prior to final entry into the blood stream where it is transported mainly as lipoproteins in small droplets of less than 1 μ in diameter called chylomicrons. When lipase activity is diminished such as in celiac disease or pancreatic disease, particularly cystic fibrosis of the pancreas, a good deal of the fat fails to be absorbed and consequently is excreted in the stools.

Fat is not only transported as tiny chylomicrons but also as phospholipids and cholesterol esters. The lipoproteins are of various molecular weights. In these differing physical and chemical states fat is transported to the body tissues.

Here again there may be derangement in this mechanism where, for some reason as yet unknown, fat accumulates in the blood and is not taken up by the tissues. One type of abnormal lipemia shows an accumulation mainly of neutral fats resulting in creamy serum, xanthomatous deposits, enlargement of the liver and spleen. Such cases suffer occasional and unexplained attacks of sudden abdominal pain with all the manifestations of an acute inflammatory process. This is followed by a rapid fall in blood fat with a simultaneous increase in the size of the liver and spleen.

The physical and chemical nature of serum fat has attracted wide attention during the last few years after the discovery of a factor present in serum that is responsible for causing decreased turbidity of lipemic sera. The so-called *clearing factor* effects a hydrolysis of the triglycerides yielding glycerol and free fatty acids. With this lipase-like action, there is a simultaneous decrease in the β lipoprotein of low density and an increase in the β lipoprotein of higher density. One of the most interesting features of the *clearing factor* is that it is activated by heparin. This can be demonstrated *in vivo* by the injection of heparin which results in rapid clearing of lipemic sera as well as causing a diminution or disappearance of low density lipoproteins. Anaphylactogenic agents also cause an increase in the activity of the serum clearing factor, presumably due to release of heparin.

Another type of derangement that manifests itself in serum is the occurrence of high cholesterol levels in the nephrotic syndrome, hypothyroidism and particularly in idiopathic familial hypercholesterolemia. In all such instances xanthomatous deposits appear in various tissues. Where hypercholesterolemia is prominent, as in the idiopathic familial syndrome, early atheromatous patches may occur similar to those encountered in arteriosclerosis.

Atherosclerosis is indeed a disease peculiar to the human species and intimately associated with cholesterol metabolism. The disease can be produced in animals by excessive feeding of cholesterol. Thus there is a definite relationship between cholesterol and arteriosclerosis even though the disease in humans is not generally associated with high blood cholesterol. Cholesterol transport is associated with the low-density lipoproteins. These are elevated in cases of atherosclerosis and appear in rabbit sera only following cholesterol feeding.

The lipid component of an atheromatous lesion is composed mainly of cholesterol and its esters. In this manner the lesions are similar in composition to those observed in the xanthomatous areas of Hand-Schuller-Christian's disease. Cholesterol is not only a constant companion of lipids in the form of cholesterol esters, but its metabolism is closely linked to lipid metabolism. The reason is not far to seek since cholesterol is built from the same elementary 2 carbon fragment active acetate (-acetyl *CoA*) that forms the smallest building block of fatty acids. Acetate labeled in both carbons is incorporated into the cholesterol molecule as a unit. Squalene has lately been shown to be an intermediate in cholesterol synthesis. Since both fatty acids and cholesterol use acetate as an essential building block, both must necessarily compete for the available active acetate. When fatty acid synthesis is suppressed, cholesterol synthesis is thereby accelerated. In diabetes not only is the synthesis of fat from acetate impaired but there is also an increased fatty acid oxidation and breakdown to yield excessive amounts of active acetate, thereby augmenting still further the synthesis of cholesterol.

The impairment of fatty acid synthesis in diabetes is not limited to synthesis from acetate. The conversion of glucose to fat is also depressed. The injection of insulin corrects this deficiency. *In vitro* studies, using liver slices from diabetic depancreatized animals, also show the same defect in fatty acid synthesis. On the other hand, synthesis is not impaired following the removal of both the pancreas and the pituitary or the pancreas and adrenals. Further evidence of hormonal effects on fatty acid metabolism is the impairment of acetate incorporation by liver slices of normal animals treated with cortisone. Impaired lipogenesis in diabetes is not due to lack of active acetate, as that is formed in excessive amounts, nor is it due to a defect in the Krebs cycle. The cause apparently lies in some deficiency in the glycolytic process. Thus impaired lipogenesis in extracts of livers from diabetic animals can be corrected by the addition of glycogen or the phosphorylated intermediates of glycolysis. However, fructose but not glucose produces the same effect. It now appears that fats are synthesized and not burned in the flames of carbohydrates since, as we have noted above, fatty acid oxidation and breakdown are accelerated in diabetes while fat synthesis is impaired. It is the latter defect that is abolished by correcting the deficiency in the glycolytic mechanism.

Fatty acid oxidation to carbon dioxide and water occurs in all tissues. However, the liver, of all organs, has a limited ability to oxidize ketones which are the products of incomplete fatty acid oxidation. These products normally are transported by the blood to other organs where they are completely oxidized. In diabetes, however, the acceleration of fatty acid oxidation produces large quantities of ketone bodies which accumulate in the blood and urine.

The steps in the complete breakdown and oxidation of fatty acids have been elucidated recently. One can picture a long chain fatty acid molecule as composed of 2-carbon units much like glycogen is composed of glucose units. Furthermore, coenzyme A (*CoA*), discovered by Lipmann, is necessary for the activation of the fatty acid molecule in much the same manner that phosphate is necessary for the activation of the glucose molecule. One further analogy is that the 2-carbon unit of a long chain fatty acid molecule splits off as the *CoA* ester, acetyl-*CoA*, much like the glucose unit of glycogen splits off as the phosphate ester, glucose-1-phosphate. The first step then in the breakdown of the long chain fatty acid molecule is the formation of the *CoA* ester. This is followed by the removal of two hydrogens (oxidation) between carbons 2 (α) and 3 (β) (R–CH$_2$–CH$_2$CO–*CoA* $\rightleftharpoons$ RCH = CHCO–*CoA*) to form an unsaturated bond. The next step involves adding water at that bond to yield the β hydroxy acid ester (R–CHOH–CH$_2$CO–*CoA*). A dehydrogenation step again follows to produce the β keto acid ester (R–CO–CH$_2$–CO–*CoA*). This keto ester reacts with a free *CoA* molecule to form acetyl-*CoA* and a fatty acid *CoA* ester with two carbons less (R–CO–CH$_2$CO–*CoA* + *CoA* $\rightleftharpoons$ R–CO–*CoA* + CH$_3$CO–*CoA*). All these steps are reversible towards synthesis and catalyzed by specific enzymes. The acetyl-*CoA* then condenses with a molecule of oxalacetate to form citrate, thereby entering the Krebs cycle where oxidation to CO_2 and water occurs.

Acetyl-*CoA* also reacts with a like molecule to form acetoacetyl-*CoA* and free *CoA*. The acetoacetyl-*CoA*, a short chain β keto acid ester, may by reversal of the above reactions become butyryl *CoA*, a short chain saturated fatty acid ester. A 2-carbon fragment can then be added onto the butyryl *CoA* by reacting with acetyl-*CoA* and again forming a β keto acid ester which by the reverse reactions forms correspondingly a saturated 6-carbon fatty acid ester. The successive addition of two such carbons yields a fatty acid molecule of the desired length. Such is the role of the active acetate, acetyl-*CoA*, in fatty acid synthesis. The first step in the synthesis being the formation of the acetoacetyl-*CoA*. This latter may, however, suffer another fate. In the liver there is an enzyme that splits the ester bond to form acetoacetate and free *CoA*. Acetoacetate can either be reduced to form β hydroxybutyrate or decarboxylated to form acetone, thus completing the assortment of the known ketone bodies. In diabetes, where the breakdown of fatty acids is accelerated, there is presumably a large accumulation of acetyl-*CoA* which forms a considerable amount of acetoacetyl-*CoA*. This cannot go toward synthesis as noted above, because in this disease fatty acid synthesis is impaired. Failing that, the excess acetoacetyl-*CoA* is then deacylated to yield excess free β keto acid and subsequently the other ketone bodies which appear in large quantities in blood and urine. These reactions occur and would seem adequate enough to explain such relevant biochemical manifestations of diabetes, nevertheless the process may be more complicated than pictured above.

The esterification of long chain fatty acids not only activates the molecules to render it available for breakdown and subsequent oxidation to carbon dioxide and water, but also to make it available for neutral fat formation by incorporation into glycerol to form triglycerides. In like manner incorporation into α glycerophosphate forms the diglyceride phosphatidic acid. The latter with the appropriate nitrogen base gives rise to the various phospholipids.

The foregoing has been a simplified introduction to the material discussed in this book. It is mainly aimed at the clinical investigator. A special effort was made to express biochemical events in simple terms with due emphasis on the main overall reactions. For details the reader is referred to the pertinent papers herein. It is hoped that this volume on *fat metabolism*, like its predecessor the volume on *carbohydrate metabolism*, will serve well its function of bringing to the clinical investigator the pertinent newer aspects of biochemistry and to the biochemist the clinical problems that have long furnished the stimulus to fundamental research.

2. Obesity in Childhood—Some Clinical Aspects

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In a clinical summary of customary excellence, Henry B. Richardson (1) writes: ". . . obesity can be regarded as a component of a neurosis, the physical expression of which is the accumulation of fat. By the latter term, I mean the bodily processes which are concerned in the obesity, including the over-all exchange of energy, and the formation, transport, storage, mobilization and oxidation of fat." He then properly poses the question as to whether these physical processes are peculiar for the obese person as compared to the person of normal nutrition. The papers to be presented in this symposium contain discussions of various facets of fat metabolism some of which may have bearing on the clinical problem.

The decision to bring the subject of obesity before you was taken by Dr. Najjar with some hesitancy. Some of his doubts must have stemmed from these considerations: (a) the concept of an endocrine etiology of obesity had been entombed with more difficulty for children than for adults, partly because obesity in childhood is frequently associated with puberty and its endocrine adjustments (2), partly because interpretation of the basal metabolic rates in obese children had been confused by choice of unsatisfactory standards of reference (3), (b) interpretable differences, in laboratory observations such as glucose tolerance, serum cholesterol, specific dynamic action as well as basal metabolism have not been demonstrated (4) possibly because of lack of studies made in the dynamic or progressive (5) rather than static phases of the syndrome. When one adds our usually fruitless therapeutic results to this essentially " negative result " metabolic literature, pediatricians are left facing their patients in a position of discomfort, one to which we are not accustomed either by temperament or experience. In spite of these intellectual and emotional considerations, the frequency of the syndrome and its continuing knottiness make it appear worthy of discussion.

It is my purpose to consider with you some clinical aspects of obesity as seen in general pediatric hospital practice, and in the pediatric endocrine and metabolism clinic of the New York Hospital where, at least during the period from 1932 to 1942, it represented the most common single cause of referral. I will not discuss some of the rarer forms such as obesity associated with:

(1) adrenocortical tumors (6)
(2) injury to the brain, e. g. encephalitis (7)
(3) a heredo-familial degenerative syndrome which may include retinitis pigmentosa, mental retardation, polydactyly and hypogenitalism (8).

In early infancy obesity develops usually without too much concern on the part of either the parents or the physician. Lack of worry stems from the recognition that good appetite and good weight are valid signs of health in babies. The hitch comes in changing opinions of what constitutes good. The earlier introduction of solids into the diets of young infants, the greater reliance on their ability to regulate the intake of artificially prepared and concentrated feeding mixtures, lead not infrequently to obesity, particularly when interpretations of hunger are made by inexperienced or compulsive mothers. Infants who ordinarily would have tripled their birth weights by the age of a year do so by age six months and this with no obviously harmful effects. One may find oneself objecting on grounds that young and skeptical pediatricians refuse to accept. Possible delay in sitting, crawling and walking do not seem important considerations. One cannot easily prove that fat infants are poorer risks when they develop croup or pneumonia or dehydration, and the fact that antibiotics control so many infections makes the house officer less concerned about the infant's obesity than he used to be when it interfered with intravenous therapy. Surgeons still would rather operate on a thin than a fat subject, but this consideration obviously should not be raised with the parents of a well baby. Parents are vicariously thrilled because they interpret the infant's ability to ingest large amounts of solids and fluids as a sign of high intelligence, not only high, but what is more important, as high as or higher than that of a neighbor's or relative's infant. It is true that most obese babies do not remain obese—their appetites and rates of gain slow down for they must remain in the human species. A certain number however remain obese and when brought to the physician later in childhood, the parent's version of early feeding habits—" he has always eaten like a pig"—constitutes quite a shift in attitude. If one has watched newly born infants, some of them premature, make sucking movements even while asleep, one recognizes the existence of early differences in orality or whatever you want to call it, if this term is unacceptable. On the other hand, the early emphasis on overeating as a pattern of gratification may contribute to the type of symptom later manifest in the neurosis of which obesity is the prominent physical sign. Although no proof for this suggestion is at hand, its possibility is suggested by the occasional recurrence of a reflex such as the whooping paroxysm in children who develop bronchitis some months after complete recovery from whooping cough.

It has seemed proper to discuss the problem of obesity with parents even during the first six months of an infant's life, not in terms of actuarial tables, but rather as a basis for inculcating the view that bigger is not better. One tries to cast at least some doubt on the benefits of conforming to mass standards either in eating habits or rates of gain and hopes it will carry over into other areas. This is actually the only basis of approach since the parents rarely complain about the infant's obesity. Pediatricians with particular experience in the field of allergy have learned to take preventive dietary steps for infants with family background of allergy (9); similar steps may be indicated for obese infants with family background of obesity.

We come now to the problem as it is seen in early, or later childhood. Parents may bring their son to the physician because they have noted that he cannot keep up with his playmates in physical activities, or because obesity makes his genitalia, which are within normal prepubertal limits, appear abnormally small. Daughters are brought by thin or fat mothers because of concern about social implications. Not infrequently obesity is found on physical examination of a child brought for some minor illness; as one takes a complete history one turns up a background in which the need for help is real, but made more complex by the fact that it is unfelt. The circumstances of development of the obesity are varied. As already stated, in many children it has been continuously present since early infancy; in some the patient was obese during the first year, thinned out for a variable period during pre-school years and then began to gain weight rapidly, occasionally after a trying experience. This is frequently a hospitalization, and the most common single cause of the latter is tonsillectomy. A child whose appetite has been poor because of chronic tonsillar infection has his tonsils removed. On return to his home he reacts to the experience with anxiety which depends on his emotional status (10) and on the success of attempts to "humanize" the hospital experience (11). This anxiety may take the form of night terrors, resumption of bed wetting, orneriness or polyphagia. To parents who have for some time been worried by their child's anorexia, the increased food intake and weight gain are most welcome. But the child who previously had in some way regulated his food intake to permit moderately acceptable increments of gain in height and weight now keeps on eating to become roly-poly, with secondary psychological and physical problems which stem from his overweight. In some children, hospitalization may require prolonged confinement, and diminished activity contributes to the positive energy balance. In others the onset of rapid weight gain is at age ten or twelve or fourteen and one sees the normally increased food intake of the adolescent converted into big spurts in height and weight, with the spurt in the latter sometimes so large as to change the subject into an obese boy or girl.

Now how does one treat these patients? Acceptance of the applicability of the law of conservation of energy (12), and of the importance of economic, cultural and psychologic factors (13) in overeating are necessary first steps in our attempts to understand these patients. Conversion of these concepts into therapeutic successes has not been easy, witness the fact that I can remember successes in detail because they have been so few in number. The histories of two recent patients illustrate some of the complexities.

The first patient, an eleven-year-old boy, was brought to the pediatric out-patient department of Sinai Hospital for evaluation of a possible cerebral birth injury. He had originally been referred for psychiatric help to a community family agency by the director of an associated camp because he did not participate in camp activities. Psychologic testing had revealed findings which might be associated with organic brain disease, and this was supported by the mother's report that the umbilical cord had been wound about his neck at birth. Troublesome symptoms at the time of examination were aggressive behavior and disobedience at home and in school, temper tantrums, poor motor skills, disinclination to play outdoors, lack of friends, an inclination to moon while sitting cross-legged for hours, a large appetite for sweets and rapid weight gain. Because of his sluggish behavior and weight gain, a physician had started administration of thyroid but without any noticeable effects. Physical examination revealed a moderately obese boy of 127 lbs., 25 lbs. overweight for his height and age, and whose genitalia were within normal limits. Neither the father or mother was obese. A review of his birth record in another hospital revealed that although the umbilical cord had been tied around his neck, his early breathing and feeding behavior suggested no perinatal cerebral anoxia. Furthermore, his early motor development had been within normal limits. The child had been cooped up alone a good deal in an apartment during his first three years because the mother helped the father in business. This was only marginally successful and the modishly dressed mother was disappointed both in this, and her son's unexceptional intellectual and physical attainments.

Although his electroencephalogram was not normal and although the mother reported that cessation of thyroid therapy had led to increased sluggishness, it was obvious that the patient required intensive psychiatric therapy not for his obesity but for the gross personality disorder. To increase this child's physical activity and decrease his nibbling will require something more than cliches. As pediatricians, we dare not probe the family situation intensively any more than we would turn down a skull flap in a child suspect of a brain tumor. We shall obtain a repeat electroencephalogram and an I^{131} thyroid uptake determination, and give some supportive reassurance while the agency arranges for psychiatric therapy. We have also recommended that sympathetic non-competitive instruction be obtained for the boy who evinced interest in improving his batting and catching skills.

The deep-seated nature of some of the primary and secondary psychologic disorders existing in obesity has been well documented. One might hope that psychiatric study and treatment of children in the early, "dynamic" phases of obesity would be profitable, but shortages of personnel and the apparent well-being of the patients mean late referral unless other overt behavior disorders are present, as in this patient.

The second patient, a 10½-year-old boy, was referred from another city in consultation. The reason for referral was headache, probably migrainous, but the mother was also concerned with her son's enuresis and obesity. The mother who had been obese, had lost 50 lbs. over a two year period, and her attacks of migraine had ceased while taking amphetamine sulphate. The patient had been moderately obese, weighing 26 lbs. at one year, but rapid weight gain had started after a tonsillectomy and move of the family to a one room apartment when the patient was three years old. His weight had recently take a 10 lb. spurt in a 4 month period of forced inactivity because of a fractured ankle incurred at camp. He was a resourceful child of superior intelligence with a full and varied schedule of curricular and extracurricular activities which included athletics. An only child, he had, unlike the first patient cited, apparently good relationships with adults, including his parents. Part of his problem stemmed from the fact that both of his parents were busy almost all of his waking hours gradually getting on their feet financially in a luncheonette business. He rustled his own breakfast from a refrigerator well-stocked with high caloric delicacies, had a concentrated lunch at school, and supper at the luncheonette. For at least part of the evening he was home alone with television and the refrigerator. Physical examination revealed a moderately obese boy of 126 lbs., approximately 40 lbs. above average weight for his age and height. Genitalia were small but within normal prepubertal limits. For this boy, his good relationships to his parents offered a base on which to build with psychiatric help. Unfortunately such help was not easily available in his community, and so we decided to temporize with a general explanation to the mother of the origin of his symptoms, prescription of amphetamine sulphate and a review of the caloric values of different foods. The child's unsolicited offer to keep a chart of his daily food intake and weight indicates an assumption of responsibility for himself that is beyond his chronological age, and while apparently laudable may represent one source of strain. If we can obtain for him supportive psychotherapy, the strain may be turned into creative productivity, particularly if the environmental situation which requires that both parents act as breadwinner improves. Recently he wrote reporting a 3 lb. weight loss, some improvement in his migraine and bedwetting and that he was trying to do this without pills, indicating that the explanation for recommending amphetamine sulphate had been inadequate.

We have gone at some length into the history of these two patients only to show the variability of the clinical pictures, and to indicate how difficult it is to translate presently available concepts of pathogenesis into practical therapeutic measures. Both patients have been seen too recently for us to have any opinion concerning prognosis.

We wish to discuss now the question of constitutional background, so well delineated by Bauer (14) and Rony (5). The familial occurrence of obesity has been repeatedly documented. This is certainly sometimes caused by family eating habits which are culturally or emotionally conditioned. We remember one family with many obese individuals in which poor economic conditions led the whole family to eat in excess the produce of the baker-father. One is hard put however, to explain the simultaneous occurrence of leanness and obesity in the same family on the basis of family eating habits. But more puzzling, how shall we explain obesity we have seen confined to the lower half of the body in both a girl and her mother who brought her to the clinic? This implies not only a constitutional factor but its expression in a regional lipophilia (5). We have no photographs of this patient and her mother, but in Rony's excellent book one may see pictures of patients with lower or upper segment obesity and finally of one patient with lipoatrophy of the face and upper trunk and adiposity of the lower half of the body. This combination of loss of fat in the upper and gain in the lower segment indicates again the importance of local tissue factors in fat deposition or mobilization, and is the basis for the original hypothesis of Von Bergmann that some genetically conditioned local anomaly of the intermediary metabolic processes in the fatty tissues of the obese patient may contribute to the development of obesity. Evidence for this hypothesis has not been brought forth, but neither do we explain patients such as the ones we have mentioned, by our customary thinking in terms of overeating and underactivity.

We have described some of our puzzlement about the clinical syndrome obesity in which there is evidence for both somatic and psychologic disorder. We conclude with a quotation from the Trimble lecture given before the Medical and Chirurgical Faculty of Maryland by Browne (15). He said: "The psychosomatic fence is a bright, white-painted picket fence on a green lawn which has no fence on it, and the medical people constantly fall over the fence on one side and the psychiatrists fall off it on the other side, and there isn't any fence there."

Lack of success in the treatment of obesity requires that we maintain a flexible attitude (16) toward these patients.

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3. Multiple Causative Factors in Obesity

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IN A RECENT REVIEW (1), it was shown that genetic, traumatic and environmental factors intervened in the etiology of obesity. While admittedly obesity will develop only if a positive energy balance is maintained for a sufficient duration, to equate the development of obesity with the maintenance of this positive balance sheds no light on the phenomenon: it simply redefines it by allusion to the First Law of Thermodynamics. In this lecture the complexity of the normal physiological devices governing food intake will be recalled. The "glucostatic mechanism of regulation of food intake" previously proposed has recently received its first independent confirmation. The multiple etiology of obesity will next be illustrated not as in the view quoted above by a systematic survey of published reports, but by examples derived from recent work, some of which is hitherto unpublished. This lecture may therefore be considered to be largely in the nature of a series of discrete preliminary "progress reports" on some of the most characteristic aspects, the logical continuity of the subject being supplied by reference to the previous review.

1. The Glucostatic Mechanism

Experimental work using rats, mice, dogs and human subjects culminated in the proposal (1–5) of a glucostatic mechanism of regulation of food intake. This postulated that the central nervous system maintains, in the lateral hypothalamus, " glucoreceptors " sensitive to fluctuations of available blood glucose and responsible for the control of appetite. A number of experiments demonstrated that in the normal or Houssay animal altering blood glucose levels modifies food intake in a predictable manner (2). The apparent paradoxes presented by the hyperphagia of diabetes mellitus, by the phenomenon of hunger diabetes, by the hyperphagia accompanying tendency to higher glucose levels in the obese, became amenable to explanation when it was noted that all these conditions had this in common, namely that while absolute levels of blood glucose were increased,

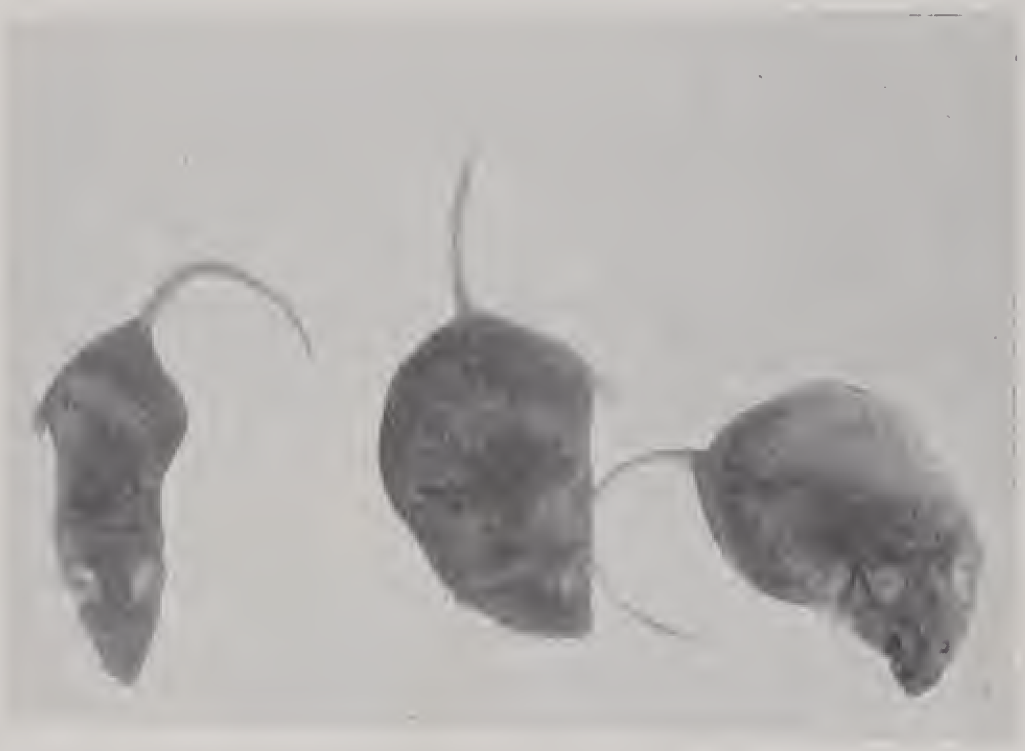

Figure 1. Illustrating the multiple etiology of obesity: a thin mouse and two obese littermates. The mouse in the midle is genetically obese, the mouse on the right hand was made obese by Goldthioglucose treatment.

utilization was decreased. For variations of blood sugar levels to influence hypothalamic receptors, glucose has to cross the membranes of these cells. This presumably implies phosphorylation through the hexokinase reaction. If phosphorylation is impaired, " effective " sugar levels will be in fact lower than the absolute values measured. Introduction of these concepts allowed the experimental interpretation of the effects of diabetes mellitus, hunger diabetes, epinephrine, cortisone, insulin, growth hormone, cold and hyperthyroidism (1, 2, 3, 5).

There is still a need for a mechanism which translates available blood glucose into variations in the physiologic state of the glucoreceptors. Such a mechanism was suggested by the observation that a drop in serum inorganic phosphate and in potassium consistently accompanies increased glucose utilization (5). It appeared possible that the passage of potassium ions into the glucoreceptors represents the point at which effective blood glucose levels are translated into an electrical or neural mechanism. This hypothesis made the mechanism vulnerable to disorders of the mineral metabolism and suggests an interpretation for Addisonian anorexia.

The concept was tested and put on a quantitative basis in a series of experiments performed on human subjects (4, 5). Because of the inaccessibility of the lateroanterior hypothalamic (" feeding ") centers, peripheral arteriovenous differences were determined as an index of rates of glucose utilization. These differences (designated as "Δ-glucose") were measured in the antecubital region (between finger blood and antecubital vein blood). With only the apparent exception of epinephrine (a hormone which decreases peripheral glucose utilization while increasing central utilization (6)) it was found that Δ-glucose values correlated closely with the caloric intake of the individual and with hunger feelings. Figure 2 (5) illustrates some of the situations

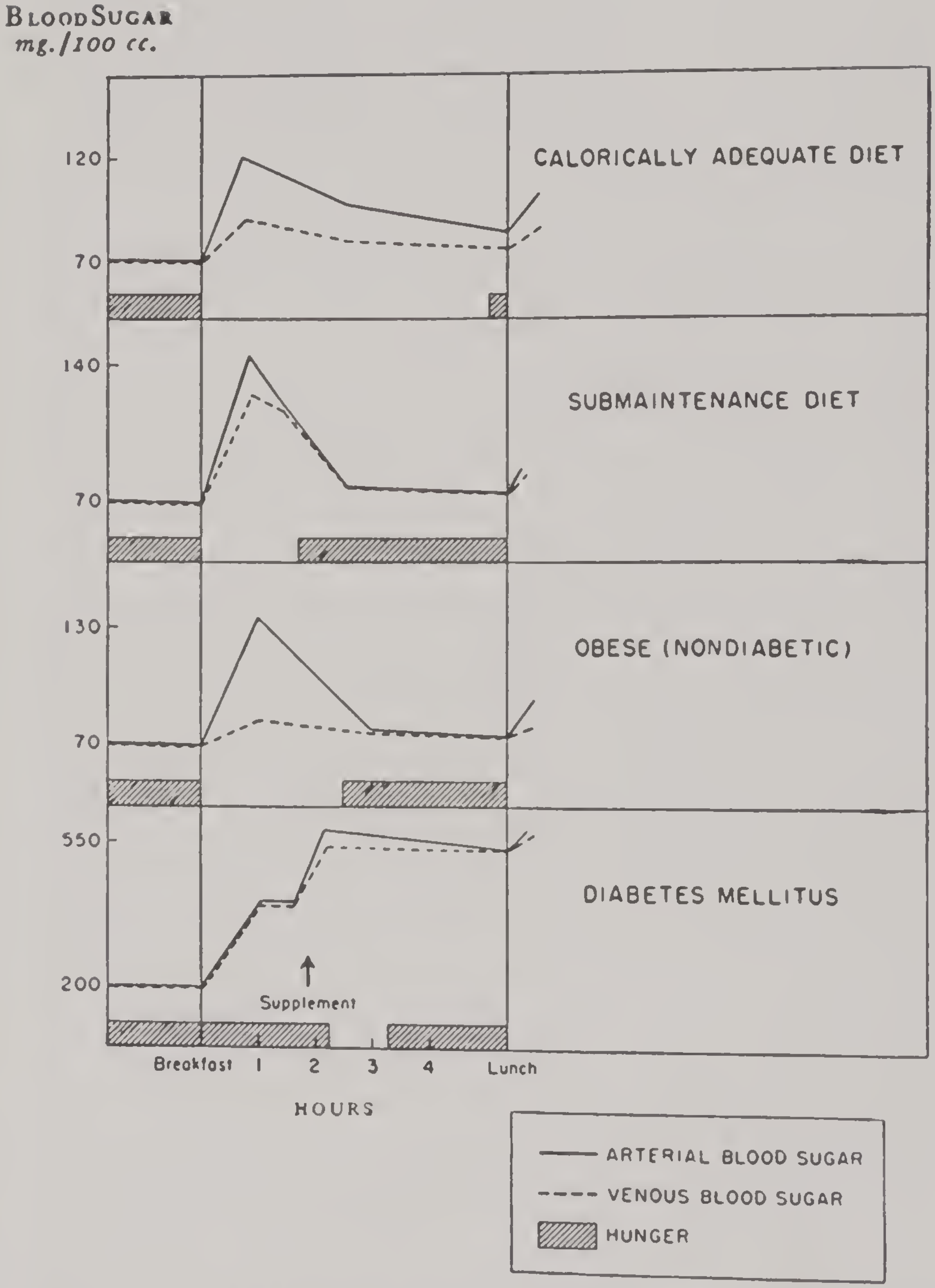

Figure 2. Typical Morning Correlations of Δ-Glucose and Hunger Feeling. The size of the Δ-glucose (peripheral glucose arteriovenous difference) correlates with hunger feelings. No hunger feeling appears if the Δ-glucose is greater than 10 mg. per 100 cc.

encountered. Calorically adequate diets were associated in normal individuals with Δ-glucose values which remains large throughout the day, decreasing only at mealtime, submaintenance diets with Δ-glucose shrinking rapidly after meals, hunger diabetes with appreciable Δ-glucose only for higher glycemias, uncontrolled diabetes with a similar (though exaggerated) picture. Obese individuals, chosen from a nondiabetic group with no diabetic familial history, and in the "active" phase of obesity, were found to be characterized by a very much increased glucose uptake (large "Δ-glucose") after meals followed by an abnormally rapid shrinking of the arteriovenous glucose difference. The effects of cortisone and of insulin in appetite are also readily interpreted by reference to similar determinations. In all cases where serum inorganic phosphorous was determined, the correlation between increase in Δ-glucose values and the drop in inorganic phosphorous was excellent (7). Recently, Dr. T. B. Van Itallie, working at St. Luke's Hospital in New York, has extended these findings to much larger groups of subjects, in particular as concerns the effect of cortisone (8).

In the studies hitherto published from this laboratory, as well as in those conducted subsequently by Dr. Van Itallie at St. Luke's, the correlation established was between Δ-glucose values and subjective feelings of hunger, as revealed by the subject. Thus, it was found that Δ-glucose values greater than 10 mg. per cent were not associated with hunger feelings while Δ-glucose values tending toward 0 for a sufficient time were associated with consciousness of hunger. Recently, Dr. A. J. Stunkard (9), working at New York Hospital-Cornell Medical School, has brought independent confirmation of these findings as well as extended them by correlating the appearance of hunger contractions of the stomach with shrinking Δ-glucose values. He used an intragastric balloon system similar to that previously used by Walter Cannon. The correlation was excellent in normal individuals as well as in some types of psychiatric disorders, while it disappeared in other mental pathological situations.[1]

On the experimental side, it has also been shown recently that excitation of the lateroanterior hypothalamic centers which, in the " glucostatic " view, represent the glucoreceptors, was followed by an increase in food intake without any detectable concomitant metabolic disturbance (10). This result, as well as those of Dr. Stunkard, indicates that these lateral (" feeding ") centers are at the origin of the cortical integration of subconscious hypothalamic impulses.

Whether the glucostatic model is eventually retained or not, it illustrates the complexity of the mechanism of regulation of food intake. It is vulnerable, in particular, to any disturbance in carbohydrate metabolism; to disturbances in fat or protein metabolism because of the sparing effect of these metabolites; to disturbances in electrolyte metabolism because of their effect on the electrical phenomena in the receptors; to increase or decrease of activity beyond the limits of adjustment of the normal regulation (see below); to faulty cortical integration of hypothalamic impulses as well as to dysfunctions of the " feeding " and " obesity " hypothalamic centers. It may be noted *pari passu* that the glucostatic scheme has the important advantage of making possible the integration of the regulation of food intake with other metabolic regulations: not only with the regulation of carbohydrate metabolism but also because of the established influence of the availability of glucose on fat utilization (11) and on gluconeogenesis (12), with the regulation of protein and fat metabolism as well. Obviously, unless such an integration were possible, the regulation of food intake could not be the precise mechanism it normally is.

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[1] Dr. A. J. Stunkard also found that in man, arteriovenous injection of glucose promptly abolished gastric contractions together with the subjective experience of hunger, *provided* glucose was properly utilized. Again expressing this utilization in terms of delta-glucose, values of the order of 20 mg. per cent were associated with this inhibition; decrease toward 0 was associated with return of both gastric contractions and experience of hunger. In the presence of both uncontrolled diabetes mellitus and hunger diabetes, intravenous injections of glucose which resulted in negligible arteriovenous glucose differences had no effect upon gastric hunger contractions or on the experience of hunger (Fed. Proc. 13: 147 [1954]).

2. Fat Metabolism in Three Forms of Experimental Obesity

In the course of a study of intermediary metabolism in the hereditary obese-hyperglycemic syndrome of mice (1, 13) it was observed (14) that when fasted obese and nonobese mice were injected with carboxyl-labeled sodium acetate in amounts proportional to body weight, the nonobese mice expired significantly (one-third) more labeled CO_2 during the 3 hours following injection than did the obese animals. When obese and nonobese animals were fed for three days doses of labeled acetate proportional to body weight, overall incorporation of labeled carbon into liver fat was more than twice as great in the obese than in the nonobese.

This experimental series suffered, however, from the following limitations: as neither the protein content (15) nor the overall oxygen content (16) are increased in the hereditary obese-hyperglycemic syndrome, it would be more logical to give obese and nonobese animals the same amount of labeled acetate, rather than amounts proportional to body weight; results would also be easier to interpret if the caloric consumption of the obese and nonobese groups were equalized; carcass lipids as well as liver lipids should be examined;

in order to clearly establish the etiological significance of the findings, it would be necessary to compare not only mature animals (which are considerably overweight) with controls of the same age but also to conduct the same comparison in young animals (where the relative fatness of the obese animals is far less marked).

TABLE 1

INCORPORATION OF RADIOCARBON FROM LABELED ACETATE INTO LIPIDS OF PAIR-FED OBESE ANIMALS AND THEIR CONTROLS

Genetic obesity (mice)	Total count in Carcass fat	Total count in Liver fat
1. Mature animals		
Obese	1232 ± 225	232 ± 88
Nonobese	903 ± 220	90 ± 27
2. Young animals		
Obese	1746 ± 470	58 ± 45
Nonobese	543 ± 231	12 ± 10
Goldthioglucose obesity (mice)		
Obese	780 ± 293	152 ± 87
Nonobese	904 ± 370	129 ± 97
Hypothalamic obesity (rats)		
Hypothalamic	1264 ± 340	100 ± 27
Controls	809 ± 259	106 ± 58

Counts reported as per cent of counts ingested × 10^3
(adapted from Bates, Mayer and Nauss, [17])

It was found in a study conducted with Miss Margaret W. Bates, (Table 1) that under these strict conditions, retention of labeled acetate in carcass fat is considerably greater in the obese mice than in the nonobese. In the mature obese animals, for instance, the number of counts retained in the total carcass lipids is more than twice that found in the nonobese. The younger animals show a total count retained three times that of the nonobese. In the case of the young animals, the order of significance is high ($p < 0.001$). In the case of the mature animals, the difference is significant at the $p < 0.01$ level. When comparing young and mature obese and nonobese animals, a significantly greater radioactive carbon retention is also found in the liver fatty acids in both groups of obese mice. The order of significance is smaller than 0.01 for both young and old animals (17).

A similar study was conducted using, instead of the Mendelian recessive condition, two types of traumatic obesity in the experimental groups: goldthioglucose obesity in mice (some of the thin littermates of hereditarily obese animals made obese by goldthioglucose treatment), and hypothalamic obesity in the rat. The goldthioglucose experiment was carried out exactly like the obese-hyperglycemic syndrome experiment. The hypothalamic rats were reduced prior to the experimental period to a weight similar to that of their controls. Obese animals were paired fed with their controls and administered the same amounts of carboxyl-labeled acetate. Table 1 clearly shows that under these conditions, no significant difference in acetate incorporation can be detected either in carcass or in liver lipids between obese animals and their controls. The hypothalamic animals retain significantly more radioactive carbon in the carcass, but not in the liver fatty acids. The order of significance between carcass acetate retention in obese and nonobese animals is high ($p < 0.0002$).

These results illustrate in a striking manner the multiple etiology of obesity: different types obviously correspond to profoundly different characteristics of fat metabolism. In the case of the obese-hyperglycemic syndrome, because of the fact that there is a much more marked difference in radioacetate retention between obese and nonobese animals in young than in mature animals (in spite of a fat content in young obese of less than half that seen in the mature obese animals), the etiological significance of the finding is emphasized. The observation fits neatly with the suggested mechanism derived from endocrine and histologic studies (1, 11, 15), i. e., a primary hypersecretion of pancreatic hyperglycemic factor causing a secondary insulin hypersecretion. The mechanism of the induction of obesity by goldthioglucose is still unknown. The acetate results give rise to one more difference between this form of obesity and the hereditary obese-hyperglycemic syndrome (see below). The findings in hypothalamic obesity differ from both those in genetic and in goldthioglucose obesity. The fact that a difference in C^{14} retention between hypothalamic animals and their controls was observed in the carcass but not in the liver gives additional evidence in favor of the previously proposed hypothesis that hypothalamic obesity is mediated through a disturbance in the sympathetic innervation of adipose tissue (1, 18, 19, 20). This hypothesis also receives support from our recent finding that hypothalamic obesity can be induced by unilateral lesion (21).

An analogous study is now in course of completion (in collaboration with Miss Margaret W. Bates and Mrs. Susan F. Nauss) on fatty acid turnover in these three forms of obesity, again using fed C^{14} fatty acids. It is anticipated that again, different forms of obesity will correspond to different patterns. In the obese-hyperglycemic syndrome, the turnover of carcass fatty acids is drastically decreased.

Similarly, in a parallel study of acetate incorporation into cholesterol, it appears that the three forms of obesity are marked by different rates of cholesterol synthesis. Preliminary results suggest that only the obese-hyperglycemic syndrome is characterized by significantly increased cholesterogenesis. It may be noted that an interesting by-product of these studies has been a comparison of the three forms of experimental obesity and of controls as regards protein, water, and fat content, as well as the nature of the carcass, liver, and brain lipids (iodine number, saponification number, lipid phosphorous content, cholesterol content, proportion of free and esterified cholesterol). Results show (15) that obese animals synthesize the same fat as do nonobese as regards all qualitative characteristics. The most atypical findings were in the obese-hyperglycemic syndrome: at a given age, the obese animals are found to contain less protein than their nonobese littermates in spite of their very much greater weight; and unlike other types of obese animals, which show a normal body cholesterol content, the obese-hyperglycemic animals show an almost linear correlation between body weight and total body cholesterol. Hypothalamic rats pair-fed with their controls show a smaller protein content and a higher fat content (22).

3. A Comparison of Two Types of Obesity Which Can Be Respectively Found and Induced in Littermate Mice

A number of forms of obesity have been studied in this laboratory. Besides the Mendelian recessive obesity in mice already mentioned (obese-hyperglycemic syndrome), goldthioglucose obesity in mice, hypothalamic obesity in rats and cats, thalamic obesity has been induced in monkeys, a dominant form of obesity (yellow obesity) has been described in mice and, recently, the author and his coworkers have studied a Mendelian recessive form of obesity in the Shetland sheep dog (characterized by normal blood glucose and cholesterol level in blood, hyperphagia and a preferential taste for fat (1, 23). Until recently, however, each form of obesity had been studied in a separate animal species, or at least in a separate strain, which made the interpretation of observed metabolic differences difficult. In the course of the past year, however, it has been found possible to induce goldthioglucose obesity in the thin littermates of genetically obese animals, (24, 25). Obesity thus induced is undistinguishable in external appearance from genetic obesity. From a metabolic viewpoint, however, the two forms of obesity seem to have very little in common except the presence of a positive energy balance in both. Even this common positive balance is arrived at through very different combinations of circumstances in the two types of obesity. Table 2 compares what is known of genetic and of goldthioglucose obesity, giving references to publications where experimental data supporting these statements are given.

4. Interaction of Constitutional and Environmental Factors in Obesity

Three such factors have received increasing attention recently: exercise, the nature of the diet, and environmental temperature.

In normal animals, it has been implied by many writers that the regulation of food intake functioned with such flexibility as to automatically respond with an increase in caloric input to an increase in energy output due to exercise. Studies conducted on adult female rats exercised for increasing durations on a treadmill moving at a speed of 1 mile per

TABLE 2

COMPARISON OF FINDINGS IN THE OBESE-HYPERGLYCEMIC SYNDROME AND IN GOLDTHIOGLUCOSE OBESITY

Object of comparison	*Obese hyperglycemic syndrome*	*Goldthioglucose obesity*
Etiology	Mendelian recessive (1)	LD_{50} of goldthioglucose (not sodium thioglucose or goldthiomalate) (1, 24)
Food intake	25 per cent above normal or less (25)	75 to 100 per cent above normal (24)
Basal oxygen consumption	Low on surface or weight basis (16)	Follows surface law (24)
Activity	Depressed to a very low degree (26)	Normal (24)
Energy	Positive through low activity, low O_2 consumption, slight hyperphagia (1)	Positive through considerable hyperphagia (24)
Food choices	Preference for high fat diets (25)	
Effect of diet on weight	Weight rapidly increased on high carbohydrate diets, decreased on high fat (29, 30)	Weight increased rapidly on high fat diet, slowly on high carbohydrate, reduced on high protein (24)
Resistance to cold	Drastically reduced (27, 28)	Normal (28)
Blood glucose levels	Generally hyperglycemic; further increased by high protein, etc. (29, 30, 31, 32)	Normal
Effect of growth hormone	Immediate and drastic elevation, even with small doses and no matter what the initial level (32, 33)	Normally resistant (38)
Effect of insulin	Extreme resistance (31, 29)	Normal sensitivity (38)
Thyroid function	Normal (41)	
Histological abnormalities	Hyperplasia of Islets of Langerhans (34, 32), degranulation of beta cells (32)	
Effect of drugs	Blood glucose, effect of insulin, growth hormone, weight corrected by diethyldithiocarbamate (32) blood glucose affected by synthalin (35)	Not known

Object of comparison	*Obese hyperglycemic syndrome*	*Goldthioglucose obesity*
Cholesterol level	High; further increased by growth hormone, high protein, etc. (30)	Normal (15)
Body composition	High in fat, low in water, (36) Protein low; cholesterol correlated with body weight (15)	High fat, low water, normal protein, no increase of cholesterol with weight (15)
Liver weight, composition	Large livers, high fat (15)	Large livers, low in fat, increased protein content (15)
Acetate incorporation in carcass lipids	Considerably increased (17)	Normal (17)
Acetate incorporation in liver lipids	Considerably increased (14, 17)	Normal (17)
Acetate incorporation into cholesterol	Increased (preliminary results)	Normal (preliminary results)
Fatty acid turnover	Decreased	
Suggested mechanism	Primary hypersecretion of pancreatic hyperglycemic factor; Secondary hypersecretion of insulin (13, 32)	Unknown
Susceptibility to disease	Alopecia, dryness of the skin, and frequent ulcers (34) no mating	Normal skin condition insufficient observation; probably no mating
	Increased susceptibility to infection of the skin (34)	No apparent increase in susceptibility to infection
	Increased susceptibility to respiratory infection (38)	
	Increased resistance to injected Ehrlich's ascites carcinoma (40)	Increased susceptibility to spontaneous tumors (39)

hour show (Figure 3) that actually, intakes vary linearly with activity only within certain limits of activity (42). Below this level (in what can be termed properly the " sedentary " range) small, increasing durations of exercise are not associated with increases in intakes; in fact a small, but significant decrease may even take place, perhaps because of better utilization of reserves (this may be related to the decrease in insulin requirements observed in exercised diabetics). Above a certain maximum, exhaustion sets in and again increasing exercise ceases to correspond to an increase in intake. The range of proportional response may well be considered that of " normal activity." Under " natural " conditions, exercise is seldom depressed to the level below which food intake becomes nonresponsive and weight increases. Exceptions are provided by domestic animals being fattened the activity of which is artificially restricted, and by many human subjects living in urbanized areas.

VOLUNTARY CALORIC INTAKE AND BODY WEIGHT
AS FUNCTIONS OF EXERCISE IN NORMAL RATS

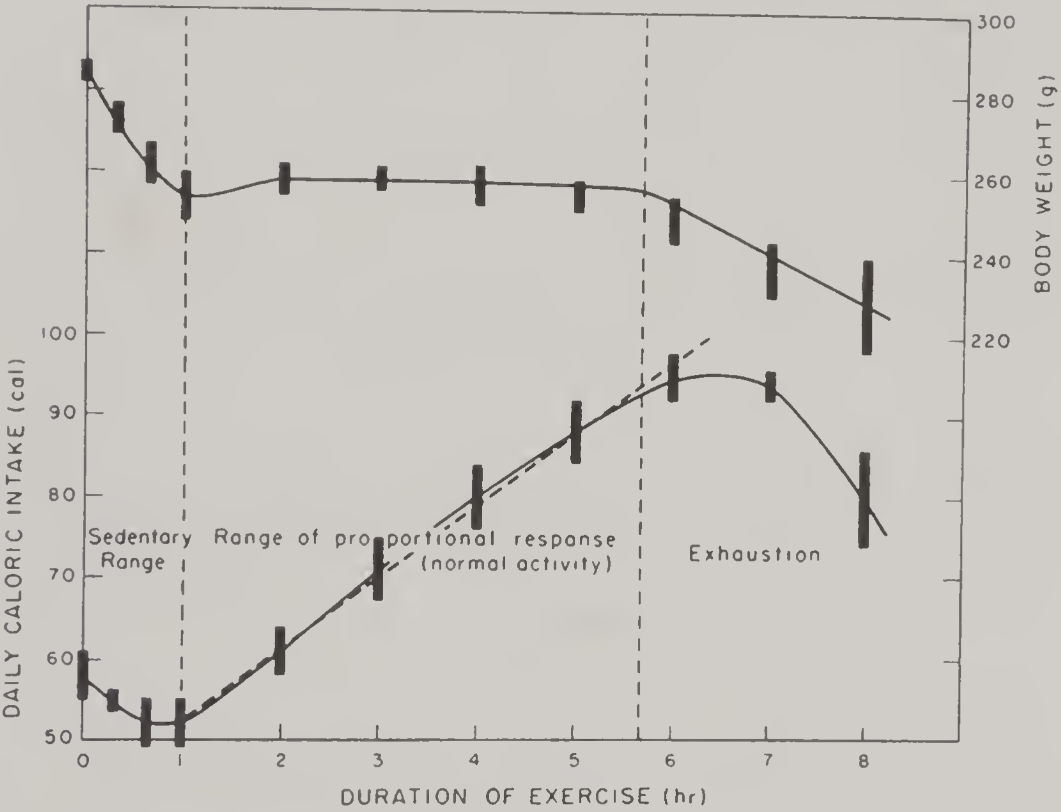

Figure 3

In obese animals, the degree of the response to exercise varies with the type of obesity considered. Mice with the obese-hyperglycemic syndrome, given an opportunity to exercise (by being made to live in an " activity cage ") will not use it (26). If they are exercised in a treadmill, their rate of weight gain is drastically reduced for a duration of exercise which leaves the weight of the nonobese mice unaffected (42). If the " waltzing gene " is bred into mice together with the obese gene, their limit weight is considerably reduced, say 40 g. instead of 100 g. On the other hand, goldthioglucose obese mice given the opportunity to exercise, will do so to such an extent that their weight is considerably reduced (24).

Exercise is doubtless an essential factor in the consideration of human obesity (43). Although fallacies to the effect that exercise represents but a small energy expenditure and is automatically followed by increased food intake have been all too widely broadcast, evidence on the role of immobilization or of sedentary life in the development of many types of human obesity is accumulating. Greene (44) has studied more than 200 overweight patients in whom the beginning of obesity could be traced directly to a sudden decrease in activity. Peckos (45) and Fry (46), working at the Forsyth Dental Infirmary in Boston, question the assertion that hyperphagia is the dominant characteristic in most obese children and incriminate decreased exercise as the essential factor in the positive energy balance leading to obesity. Our own results, obtained in cooperation with Miss M. L. Johnson (47) on high school girls in Brookline and Newton (Mass.) show definitely that the average caloric intake of these obese subjects is no greater than that of their nonobese controls of similar age, height and school grade, but that their physical activity is considerably less.

Reactions of obese animals to diets of various compositions depend on the type of obesity considered. For example, hypothalamic hyperphagic rats show the greatest caloric intake and weight on a high fat diet, a greater than normal consumption on a high carbohydrate diet, while on a high protein diet they gain little or no weight (48, 49, 1). Goldthioglucose obese mice show a similar picture (24). By contrast, mice with the obese-hyperglycemic syndrome gain weight at the maximum rate on a high carbohydrate diet, gain slowly on a high protein diet and lose weight on a high fat, carbohydrate-free diet (29, 30). Even in "normal" animals, the effect on body weight of switching from a high carbohydrate to a high fat diet depends on the strain considered, most animals showing only a temporary increase (50) while others show a permanent change (51). P. F. Fenton has shown that there was a real phenomenon of "luxus consumption" in certain strains which increase their energy output on a high fat diet, while others do not increase their intake or do not increase their output and become fat (51).

Temperature gives rise to similar observations relating to interaction of environmental and constitutional factors. It had long been known that normal animals increased their food intake with decreasing environmental temperature (within certain limits) and at a rate depending on the species, the strain and the individual (53, 54). Recent work demonstrates that cold has a profoundly different effect on different forms of obesity, hypothalamic and goldthioglucose being characterized by great ability of adaptation to cold (55, 20, 28) while the obese-hyperglycemic syndrome is not (27, 28).

Knowledge of the effect of the nature of the diet and of cold on different types of human obesity is rudimentary, because of the primitive state of "typing" in human obesity (1). No consistent deviation of pattern from normal habits can be obtained on a population of obese individuals by controlled conventional methods of inquiry (56, 57, 1). There is a widespread belief that high protein diets have the same effect in man as they have in goldthioglucose and hypothalamic obesity. High fat, low carbohydrate diets similar to those on which obese-hyperglycemic mice lose weight have also been advocated (58). Similarly it may well be that different types of human obesity respond to cold by different degrees of increase in food intake. Controlled observations supporting the belief, widespread in many cultures, that either cold shower or turkish bath favor fat mobilization have not been obtained, at least as far as the author knows.

5. The Extent of Our Ignorance of Human Obesity

In many cases, the limits of our knowledge are recognized gaps or borders which slowly recede before experimental work. In the case of studies on obesity, the obstacle has been largely of a different nature: the all too prevalent belief that we *do* know the cause of obesity and that therefore, no research is needed on this point. Obesity, too many workers believe, is *explained* by overeating; actually, it should be recognized that this is simply restating the problem in a different way, and reaffirming (somewhat unnecessarily, more than a century after Robert Mayer and Joule) one's faith in the First Law of Thermodynamics. To " explain " obesity by overeating is as illuminating a statement as an " explanation " of alcoholism by chronic overdrinking.

The abdication of physiologists and biochemists in the search for the mechanism of hyperphagia, and of excessive lipogenesis, and of positive energy balances has resulted in a plethora of uncontrolled psychological studies of obesity and unsupported psychological theories purporting to explain obesity through oral gratification, fear of pregnancy, desire for pregnancy, feeling of importance, excessive timidity, desire for protection against risks, desire for intimidations of enemies, etc. Ironically, many of the most pretentious theories have been based on " facts " which collapse under controlled observations, such as excessive intake of carbohydrates (or of fat), constant partaking of small meals (nibbling) or enormous intakes at one sitting, none of which can be established on a statistical basis (57). Similarly parental disagreements often implicated do not seem to have statistical significance (59). Even "excessive" intake per se is in some situations a less important factor as compared with decreased activity (45, 46, 47). All too often, even when psychological abnormalities are established by controlled studies, failure to distinguish between causation and association is encountered (1).

This lack of understanding that obesity, like any other pathological condition, is due to genetic, traumatic and environmental factors and to their interaction, and the refusal to recognize that we know very little of the physiological (and psychological) aspects of the problem explains the failure of present-day methods of therapy (60). The lack of anthropometric (1, 61) and metabolic (62) typing of the patients further decreases the chances of successful study and treatment. That more than "instructing" and preaching to obese patients is needed if the problem of obesity is to be conquered, is well illustrated by a recent statement of this excellent student of the psychiatry of obesity, Dr. Hilde Bruch, who finds that in many of her obese patients, the real psychological disturbance is "a compulsion to be thinner than their natural make-up demands." She writes: "in many of my cases, the conflict of not being allowed (by cultural pressure) to be one's physical self becomes the crystallization point for many other difficulties of living" (63). It thus seems that, ironically, psychiatrists are emphasizing the need for physiologists to study the constitutional and enviromental factors determining one's physical self and in particular the presence and degree of obesity. I think that it will be agreed that this emphasis from psychiatrists further eliminates any excuse for complacent statements about the physiological mechanism of human obesity. It should stimulate more experimental, clinical, and epidemiological work on the etiology of the various forms of human obesity.

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DISCUSSION

3a. Constitutional Factors in Obesity

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That there is some inherited propensity for obesity seems self-evident. Everyone has had experience with families in which the condition seems as characteristic of the family as leanness appears to be characteristic of others, but the genetic mechanism of the condition remains obscure, and there are no well characterized single gene effects associated with obesity among humans as there are in experimental animals.

The efforts to date to elucidate the constitutional element in obesity have been of three types: (1) The study of pedigrees which seemed to show obesity or a characteristic distribution of fat as a segregating single gene effect. (2) The study of the familial distribution of obesity in families of index cases. (3) Twin studies.

In the first instance there are a few syndromes which are rare and poorly understood in which obesity seems to be associated with other conditions, and which may appear in one or more members of a sibship. For example, there are (a) the Laurence-Moon-Biedl syndrome; (b) Morgan's syndrome, in which obesity is associated with hyperostosis of the frontal bone; and (c) macrosomia adiposa congenita, a condition in which babies of normal birth weight begin immediately to grow very rapidly and become enormously obese.

Among the peculiarities of distribution of fat is often mentioned the steatopygia of some African ethnic groups. Because most of these conditions appear in one generation only they are said to be caused by a single gene segregating as a recessive, but there seems little precise evidence for this.

The second type of study has made use of index cases of obesity, and the incidence of obesity in parents and children was determined. The studies, notably those of Bauer (1), Gurney (2), Rony (3), and Angel (4), indicated that an obese individual was more likely to have obese parents, sibs, and offspring than a non-obese. The fact that sibships consisted of some obese and some normal weight or lean individuals, and that matings of two obese individuals produced more fat children than matings of one obese and one normal weighted individual; and these latter produced more than matings of two normals, was taken as showing some segregation, though the figures by no means fit any Mendelian hypothesis. In all of these studies, only body weight was measured, and that this statistic may not accurately measure body fat has been shown by Keys (5), who has indicated that individuals of the same weight may vary widely in fat content. Furthermore, all the investigations are subject to the statistical bias inherent in the index case method, and I know of no population study which has been done to avoid this. The work does, however, suggest that there is a constitutional element in obesity, though it says nothing about genetic mechanisms. It would seem also to indicate that there are a good many genes involved rather than only one or two. How much of obesity is due to environmental factors common to families or specific for obese individuals is not indicated by these studies.

The third type of investigation concerns twins. Newman, Freeman, Holzinger and colleagues (6) and von Verschuer (7), studied the correlation of weights of identical twins, fraternal twins, and their sibs. The correlation coefficients were highest for identical twins, while those for fraternal twins and sibs were lower and about equal. However, identical twin pairs reared separately showed rather lower correlation coefficients, indicating that environmental effects were certainly appreciable.

In general, in order to determine a single gene effect, one needs a discrete and measurable difference, a bimodality or plurimodality in a population. If we assume that each gene has a discrete chemical effect, such measurable differences must be present in individuals carrying different genes at the same locus; but this becomes difficult when dealing with continuous variables such as obesity, and particularly, when the variable is so largely subject to environmental influences. What is needed as a first step in the elucidation of the genetical control of obesity is (1) accurate measurements of fat content applied to families and (2) accurate measurement of physiological or biochemical functions which may be specific for the individual and which may show family patterns.

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3b. Endocrine Factors in Obesity

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I am sure that we all agree that every case of obesity is due to a caloric intake in excess of the energy and growth requirements of the individual. I am delighted that for the first time the experiments of Dr. Mayer substantiate scientifically that this caloric excess may arise from a number of different causes. In some of his rats there is excessive appetite and food intake; in others there is obvious decrease of energy expenditure. The same is true in children. In one group of patients there is very obvious *overeating.* This may be due simply to overindulgence and habit, or there may be psychogenic factors such as described by Hilde Bruch. Very *occasionally* the question arises as to whether a voracious appetite could be due to a lesion in the hypothalamic centers which control appetite. (These cases must be exceedingly rare and I shall refer to them later.) In the other group of cases, obesity develops in spite of the fact that the child may not eat any more than other children of the same age, height and sex. In these cases, I believe that there is *decreased expenditure of energy* rather than some mysterious anomaly of fat metabolism. This decreased expenditure of energy is not revealed by the B.M.R. test but depends upon subtle differences in the patient's whole mode of life causing decrease of the total energy expended each day. The most grossly decreased requirements are seen in patients with extensive flaccid muscular paralyses, muscular dystrophies or amyotonia congenita. An exceedingly obese patient with amyotonia is shown in Figure 1. When these are not present and when obesity still occurs in the absence of increased intake, one is inclined to attribute decreased caloric requirements to "*constitutional factors*" (as shown in the photo of twins, Figure 2). I believe that these constitutional factors are concerned with the whole psychologic personality, neuromuscular co-ordination and entire mode of life of the individual. A large group of the fat boys we see are "constitutionally" the same. They start out by being clumsy, awkward children lacking in the finer co-ordination or the muscular drive which would enable them to enjoy the rough play, sports or athletics of the average boy. They are often superior intellectually. Accordingly, they take to a relatively sedentary life—television, reading, music, painting, etc. Even on a normal diet they become fat because of their decreased energy requirements. They are often accused of being "sissy."

I agree that the endocrine glands cannot be implicated in obesity with the very rare exception of Cushing's syndrome. Of about 200 children with definite hypothyroidism, only two were obese. The reason for this is that the hypothyroid patient has an exceedingly small appetite. They may be overweight due to myxedematous fluid but are not obese. Pituitary disorders are not associated with obesity.

The diagnosis of Froehlich's Syndrome or of adiposa dystrophia genitalis has been grossly abused. In 18 years I have seen only one or two cases corresponding to Froehlich's famous patient, who actually was not grossly obese. Among many hundreds of fat children, I know of one or two who died rather suddenly and mysteriously and were shown, at autopsy, to have microscopic changes in the hypothalmus.

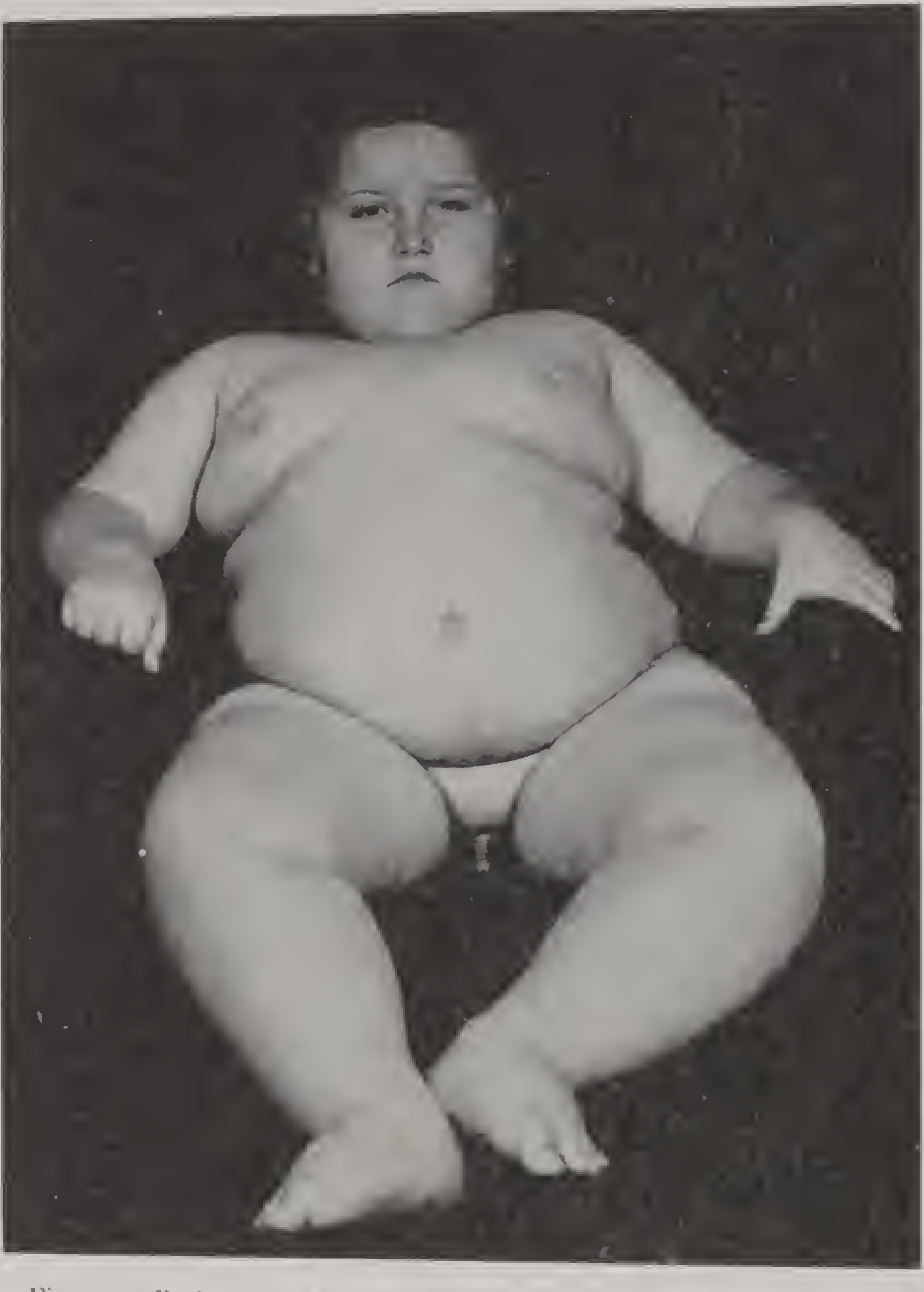

Figure 1. Patient was 6½ years old, 48 inches tall and weighed 116 lbs. (an excess of 176%). He was almost completely paralyzed due to amyotonia congenita with marked atrophy of the muscles and had been bedridden all his life. He gained weight excessively on 800 calories a day. It is probable that because of the extensive muscular atrophy his energy requirements were less than 500 or 600 calories. (From Wilkins, L., *The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence*, Thomas, Springfield, Illinois, 1950.)

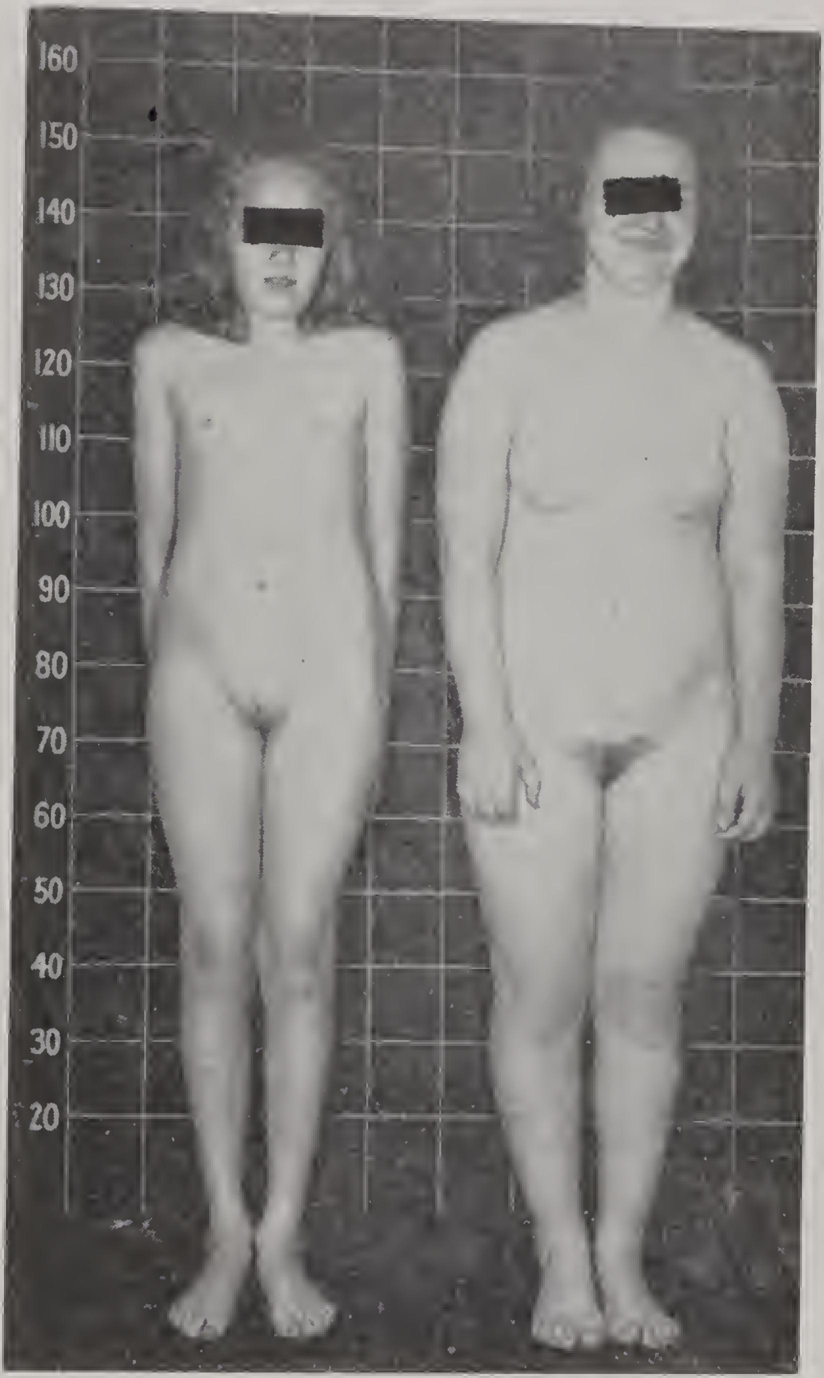

Figure 2. Twins at age of 12

Weight 87 lbs. Weight 143 lbs.

These twins show marked constitutional differences in their physical habitus, pattern of adolescense, psychic temperament and tendency to obesity. There is little difference in their dietary habits but probably a marked difference in the expenditure of energy in their daily activities. (From Wilkins, L., *The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence,* Thomas, Springfield, Illinois, 1950.)

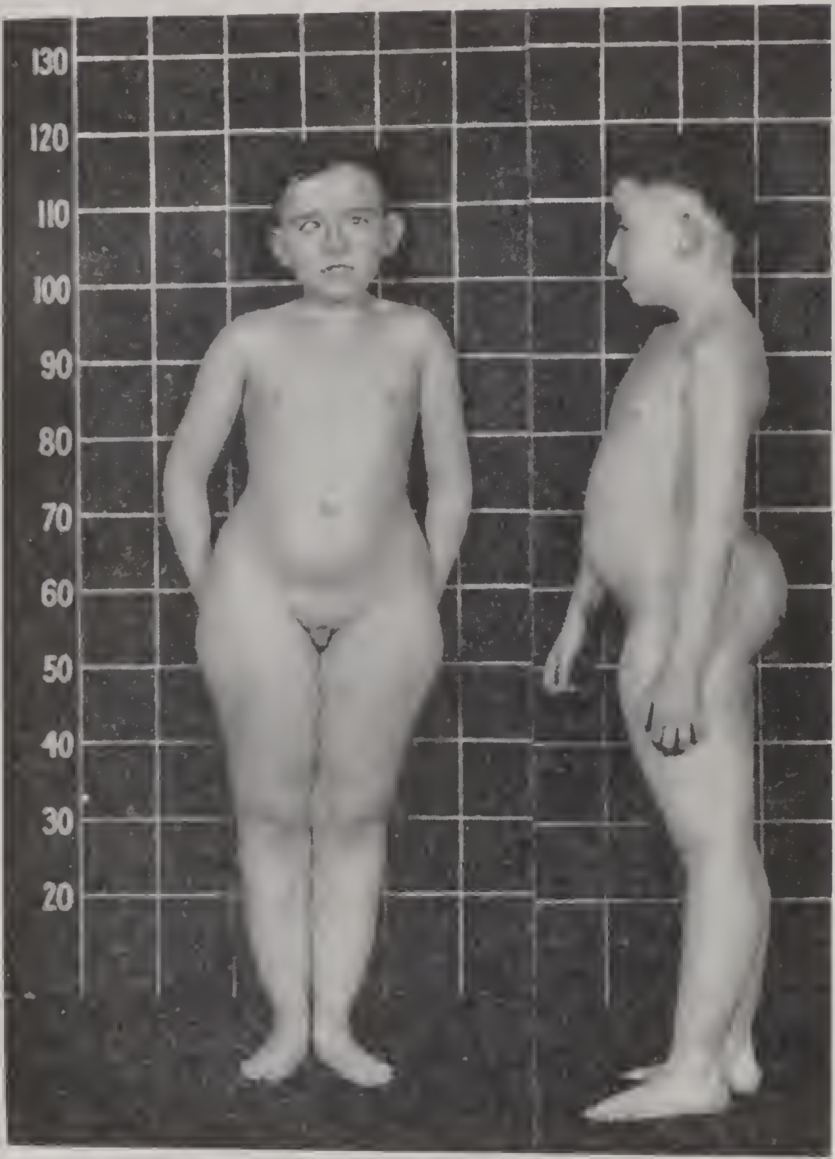

Figure 3. Age 10 years, height 46.2 inches, weighed 66 lbs., an excess of 30% for his height. A low calorie diet (875 cal.) with 110 gm. of protein had reduced his weight from 89% excess but caused wasting and weakness of the muscles. Excessive fat deposits persisted over his hips and thighs, while the upper part of his trunk, face and arms became relatively slender. (From Wilkins, L., *The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence*, (Thomas, Springfield, Illinois, 1950).

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One of these had had irregular unexplained hyperthermia for a long period. In addition, we have seen perhaps five or six obese boys who closely resembled each other. They all had difficult births and were very hypotonic and inactive in early infancy so that they were suspected originally of having amytonia. They were slow in motor development, poorly co-ordinated, moderately retarded in growth, osseous development, and mental development but eventually attained low-normal mentality. They had lax ligaments and joints and had bilateral cryptorchidism. They were relatively inactive physically and had very large appetites but no diabetes insipidus. They became progressively more obese. I suppose that this could be called adiposa dystrophia genitalis. I see no reason to blame the endocrine glands. Whether it represents a congenital anomaly of development, involving the neuromuscular system and perhaps the hypothalamus I do not know. Nor is it clear whether the obesity is due merely to the very sedentary life or to increased appetite dependent on a hypothalamic abnormality. Patients in this group are exceedingly difficult to reduce, even when the caloric intake is maintained at 600–800 calories per day. Under these circumstances, I have seen several who maintained depots of fat, while their musculature seemed to waste (Figure 3). I wonder whether in these rare cases there can be a tendency to hold fat in the depots and to burn protein selectively.

4. Lipemia

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My remarks today will be purely at the clinical level. The mechanisms of fat transport and of fat utilization, aberrations of which are responsible for lipemia, will be discussed later by several people who know considerably more about the subject than I do. I shall confine myself to giving a brief survey of the clinical facts with some detailed observations of an unusual type of lipemia that we had an opportunity to study in the Harriet Lane Home some eighteen years ago.

Lipemia--or more properly "hyperlipemia"—may be said to be present when the total blood lipids exceed 1 per cent. The excess is present in the plasma, not in the cells. One can classify lipemias in several ways: (1) by the mechanism responsible for the condition, (2) by the lipid fraction and (3) by the associated clinical condition. Table 1 shows

TABLE 1

MECHANISMS PRODUCING LIPEMIA

1. Excessive intake—*alimentary lipemia*
2. Abnormal mobilization from depots—*transport lipemia*
3. Defective removal from blood—*retention lipemia* (Fatty liver not conspicuous)
4. Defective utilization after removal from blood—*obstructive lipemia* (Fatty liver marked)

the possible mechanisms which may be at fault. An *alimentary* lipemia presents no difficulty of recognition, nor does an *obstructive* lipemia if a markedly fatty liver can be demonstrated. Lipemias due to excessive mobilization of fat from the depots (*transport* lipemia) or defective removal from the blood (*retention* lipemia) are less readily identified.

A classification by the lipid partition also has its drawbacks. Elevation of one fraction is invariably accompanied by some degree of elevation of the others, and even in the same clinical entity one finds a considerable variation in the proportion of the different lipids. Nevertheless, a rough separation is possible on the basis of whether the elevation is predominantly in the neutral fat or the cholesterol and such a classification I have set down in Table 2.

As a rule the phenomenon of lactescence—" milky serum " —is more conspicuous in the lipemias due predominantly to neutral fat than in those due predominantly to cholesterol and cholesterol esters. In the latter lipemia may be to a considerable extent " masked." Instances are reported in which the blood may contain more than 3 or 4 per cent of lipids and yet show no lactescence. The factors controlling particle size which is responsible for lactescence are complex; bile salts, lipoprotein combinations and the proportion of phospholipids to other fractions are concerned. Dr. Anfinsen has studied this phenomenon and will doubtless say something about it.

Symptoms resulting from lipemia differ to some extent in the different clinical entities. Massive xanthomatous lesions about the tendons and joints are characteristic of idiopathic familial hypercholesterolemia and are seen exclusively in that condition. In nephrotic and hypothyroid hypercholesterolemia skin xanthomata are described, but are very exceptional. Early atheromatous change is seen in all the hypercholesterolemic group. In the " neutral fat lipemias," symptoms may be observed when the blood fat reaches a high level; the retinal vessels have a creamy pink color as they emerge from the disc which becomes a pure cream as they approach the periphery of the fundus (lipemia retinalis); cutaneous xanthomata are found very commonly in this group; enlargement of the liver and spleen may also be found (1). In some subjects there are acute abdominal attacks which will be described below. A relation between this form of lipemia and early atheromatous change is not clearly established.

TABLE 2

CLINICAL CLASSIFICATION OF LIPEMIAS

I. *Predominantly Hypercholesterolemia*
   1. Nephrosis: Nephrotic Nephritis
   2. Hypothyroidism
   3. Some forms of Liver Disease (? Biliary Cirrhosis)
   4. Idiopathic Familial Hypercholesterolemia

II. *Predominantly Neutral Fat*
   1. Alimentary Lipemia
   2. Liver Disease
      Poisons: $CHCl_3$, CCl_4, C_2H_5OH, P
      Von Gierke's Disease (infants and young children only)
      Niemann-Pick Disease (rarely)
      Anoxia?
      Inanition
   3. Diabetes Mellitus
   4. Pancreatitis Acute or Chronic
   5. Pregnancy
   6. ACTH or Cortisone Therapy
   7. Idiopathic Familial Lipemia

I am not prepared to state why lipemia occurs in the various clinical conditions listed. There are theories, but little clear-cut evidence. One can postulate that the disturbance of carbohydrate metabolism in diabetes and that produced by intensive administration of ACTH or cortisone results in an abnormal call for depot fat and that the lipemia is essentially one of transport or mobilization. In many instances mixed factors are probably responsible; in the case of the liver poisons the parts played by abnormal mobilization due to deranged carbohydrate metabolism and by direct interference with fat utilization are not clearly separated. The multiplicity of lipotropic factors now known adds to the difficulty.

I shall take a few moments to mention briefly observations made when I was in Baltimore on two varieties of lipemia—alimentary lipemia and idiopathic familial lipemia. Alimentary lipemia is a matter of no clinical importance. We were, however, interested in ascertaining whether the mechanisms concerned in removing fat from the blood were adaptive or not. In the case of carbohydrate it is well known that the ability of the organism to remove glucose from the blood is conditioned by the carbohydrate load. A carbohydrate-poor diet gives rise to the condition known as Hofmeister's diabetes—a diabetic sugar tolerance curve in response to a normal load, a phenomenon which disappears quickly when an adequate carbohydrate intake is given. Observations by R. J. Myers and myself made on several normal infants in the Harriet Lane Home showed that a similar phenomenon occurs in the case of fat. After one week of generous fat administration the alimentary lipemia disappeared.

Our observations on idiopathic familial lipemia were also made in the Harriet Lane Home some eighteen years ago. The entity had been previously reported in the German literature, but not described in detail. Drs. Timbres, Aylward, and I had the opportunity to study an eleven-year-old girl who had suffered from the age of four from intermittent attacks of acute abdominal pain with projectile vomiting and fever. Quite accidentally it was discovered that she had an extreme degree of lipemia (Table 3), and examination also revealed hepatomegaly and splenomegaly. All the usual causes of lipemia and of enlargement of the liver and spleen were excluded. In the course of her stay in the hospital, we had an opportunity to witness five of these abdominal attacks. They were dramatic. The abdomen was tender and showed board-like rigidity. There was fever and leukocytosis. We were perplexed at what was happening until it was observed that the attack was associated with a remarkable clearing of the lipemia. The blood fat which had attained a level of 7 to 8 per cent prior to the attack fell at a rate as rapid as 1 per cent per hour, reaching a low level between 1 and 2 per cent in the course of eight or ten hours. The lipemia retinalis disappeared entirely and there was a striking increase in the size of the liver and the spleen (Figure 1). These phenomena were repeated with each attack. We tried many things to influence the attack: the then-known lipotropic agents: lecithin, choline and the debatable " lipocaic " of Dragstedt; we tried a number of hormones: insulin, thy-

TABLE 3

PARTITION OF SERUM LIPIDS

	Total fatty acids %	Phospholipids	Total cholesterol %
Normal values	.19– .42	.17–.33	.10–.23
Patient C. B.	.93–7.80	.15–.45	.18–.40

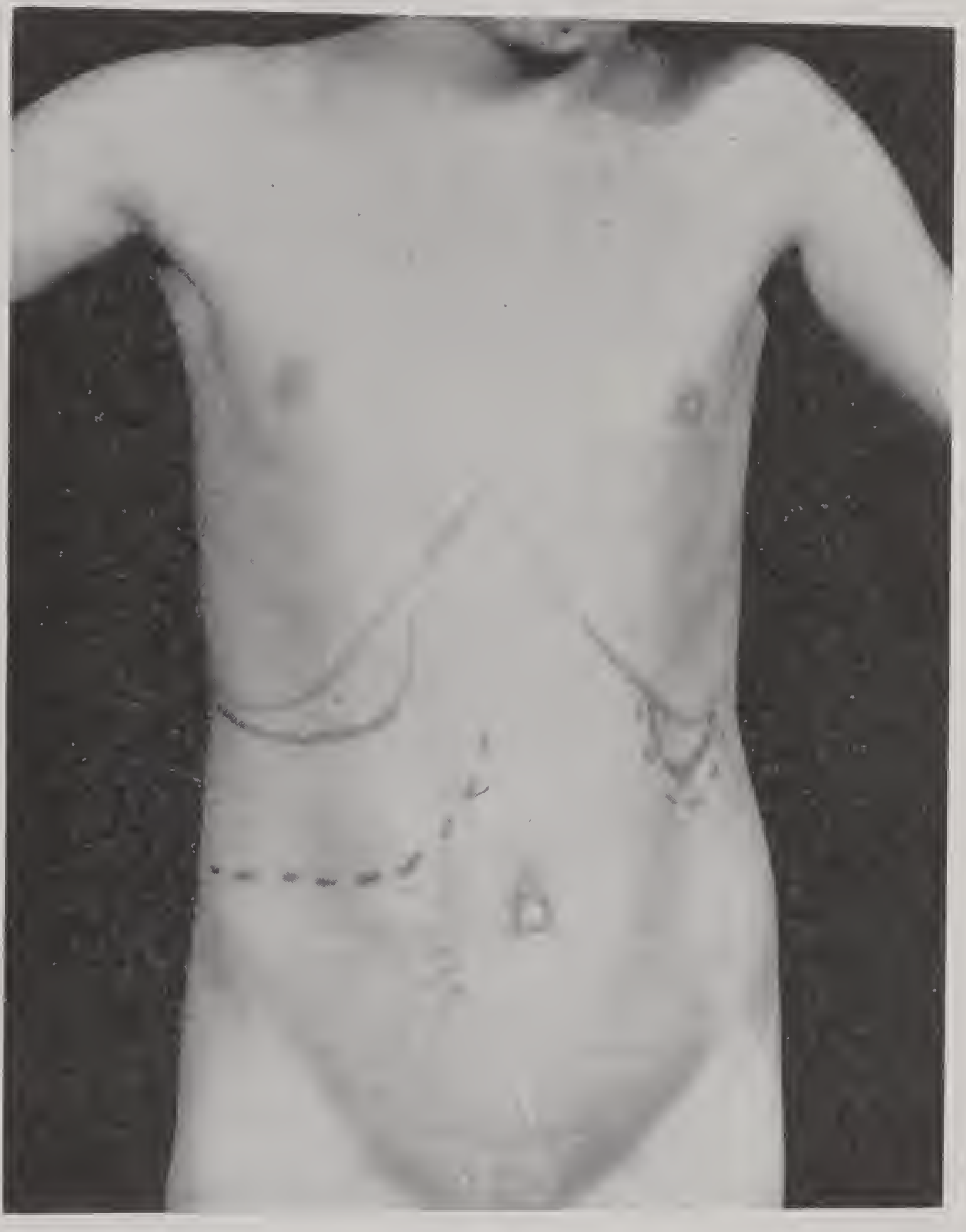

Figure 1.

roid and anterior pituitary preparations, as well as liver and blood transfusions. The only effective means of controlling the attacks was a low-fat diet; when this was rigidly adhered to the blood fat could be kept between 2 and 3 per cent, a level at which no attacks occurred.

An investigation of the family revealed one sibling with a similar condition—a lipemia in the neighborhood of 3 per cent with hepatosplenomegaly; the mother had a "forme fruste." The exact nature of the abdominal attacks was never established beyond question. Our interpretation was that we were dealing with a "retention lipemia," a difficulty of removing fat from the blood by the liver: when the blood fat concentration rose to a sufficient level an accessary mechanism for fat removal came into play, namely phagocytosis by the Kupffer cells of the liver and phagocytes of the spleen. The resulting distention of the capsule of these organs gave rise to the abdominal pain.

In the eighteen years that have elapsed since our report, considerably more information about this interesting condition has been obtained. Some twenty-two cases have been reported in the literature and many more unreported are being observed. In one clinic alone, ten of these patients are being followed. The clinical triad of cutaneous xanthomata, hepatosplenomegaly and intermittent abdominal pain has been regularly observed, and the lipemia has affected primarily the neutral fat fraction. The most recent publication on the subject by Joyner (3) points out that when adults are affected by this condition they often have chronic low-grade abdominal pain rather than acute attacks and that when they have acute attacks there is little change in the size of the liver and spleen. Why there should be such a difference between the childhood and adult picture is not clear. Up to the present time there are no reports of patients who have been followed into adult life. It is therefore of considerable interest that Dr. Ahrens at the Rockefeller Institute Hospital has recently had an opportunity to restudy our original patient as an adult. He is fortunately here today and can tell you about his observations first hand.

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4a. Essential Hyperlipemia

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It is a privilege to be able to present on this occasion a follow-up report on Dr. Holt's patient 19 years after his classic study. The patient is now 29 years old, a housewife and factory worker. Perhaps because she has never adhered faithfully to the low-fat diet repeatedly prescribed for her, she has continued to have numerous attacks of abdominal pain of varying degrees of severity. Most attacks have been minor and have been characterized by upper abdominal soreness, nausea and anorexia, but at least three have been attended by shock and have required hospitalization. On one such occasion, 3 years ago, she was operated on with the preoperative diagnosis of ruptured abdominal viscus. The surgeon's description of the findings merits the following partial quotation: there was " an extensive collection of chylous ascites " exuding from a " small area behind the ligament of Treitz. The lacteals were all distended." There was " a retroperitoneal mass somewhat higher than the pancreas " which was not explored. The pancreas, liver, gall bladder and spleen were grossly normal, and no biopsies were made. The nineteen-year-interval history is otherwise unremarkable, except for the notation that she has had no symptoms which suggest arteriosclerotic disease.

When she was admitted to The Hospital of The Rockefeller Institute in September, 1953, for metabolic study, she was found to be a small but well developed young woman.

The soles of both feet showed eight or ten yellowish, slightly raised nodules which on biopsy proved to be xanthomata. The patient had been aware of these lesions for only a few weeks. There was typical lipemia retinalis, but no xanthelasma or tendon xanthomata. The liver was palpable only on deep inspiration, and the spleen was not felt. Duodenal drainage with secretin stimulation showed normal pancreatic function. Electrocardiograms were normal.

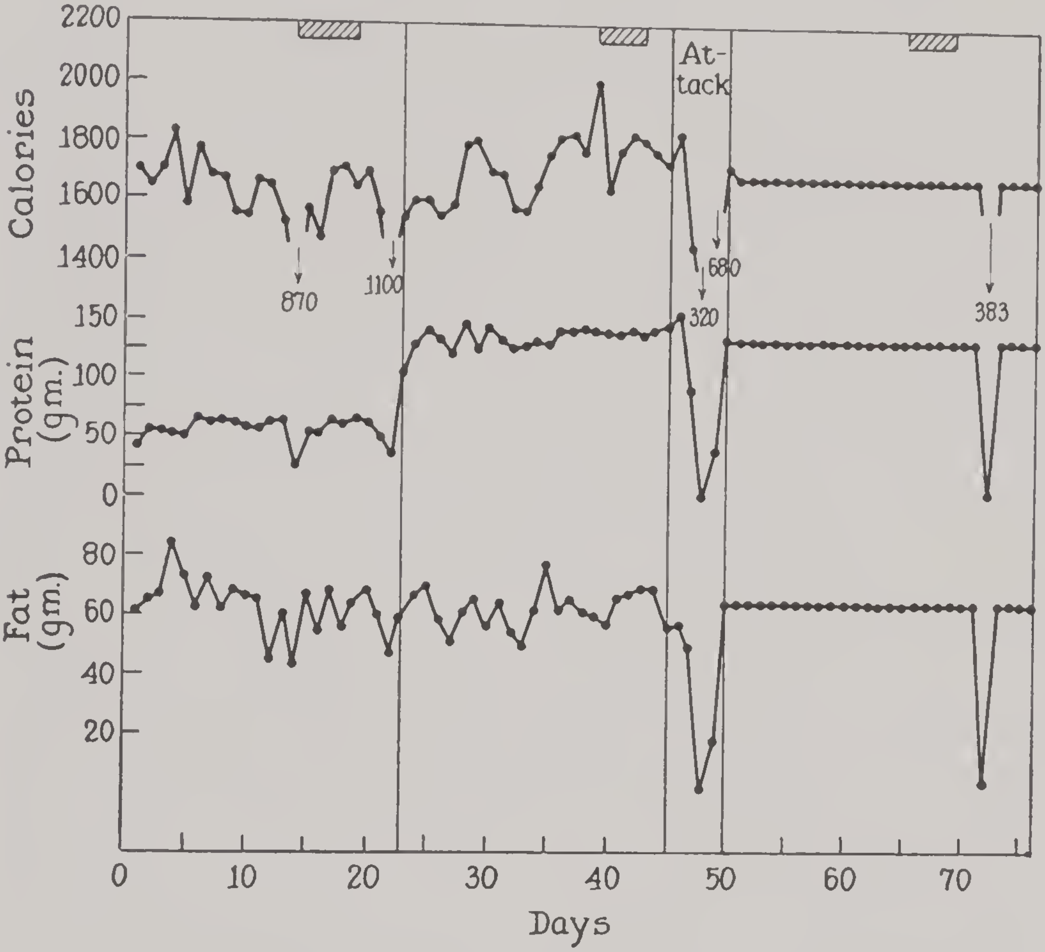

Figure 1. Food intake calculated in metabolic kitchen from standard tables. Attack of abdominal pain described in text. Formula feeding from 50–77th days, except for 36 hour fast at 72nd day. Shaded rectangles represent menstrual periods.

Our two-and-one-half-month study is summarized in Figures 1 and 2. The patient selected a diet containing

63 gms. of fat (60 gm. animal and dairy fat, 3 gm. plant fat) and was maintained at this fat intake throughout the study. For the first 22 days she ingested 58 ± 10.2 gms. of protein,

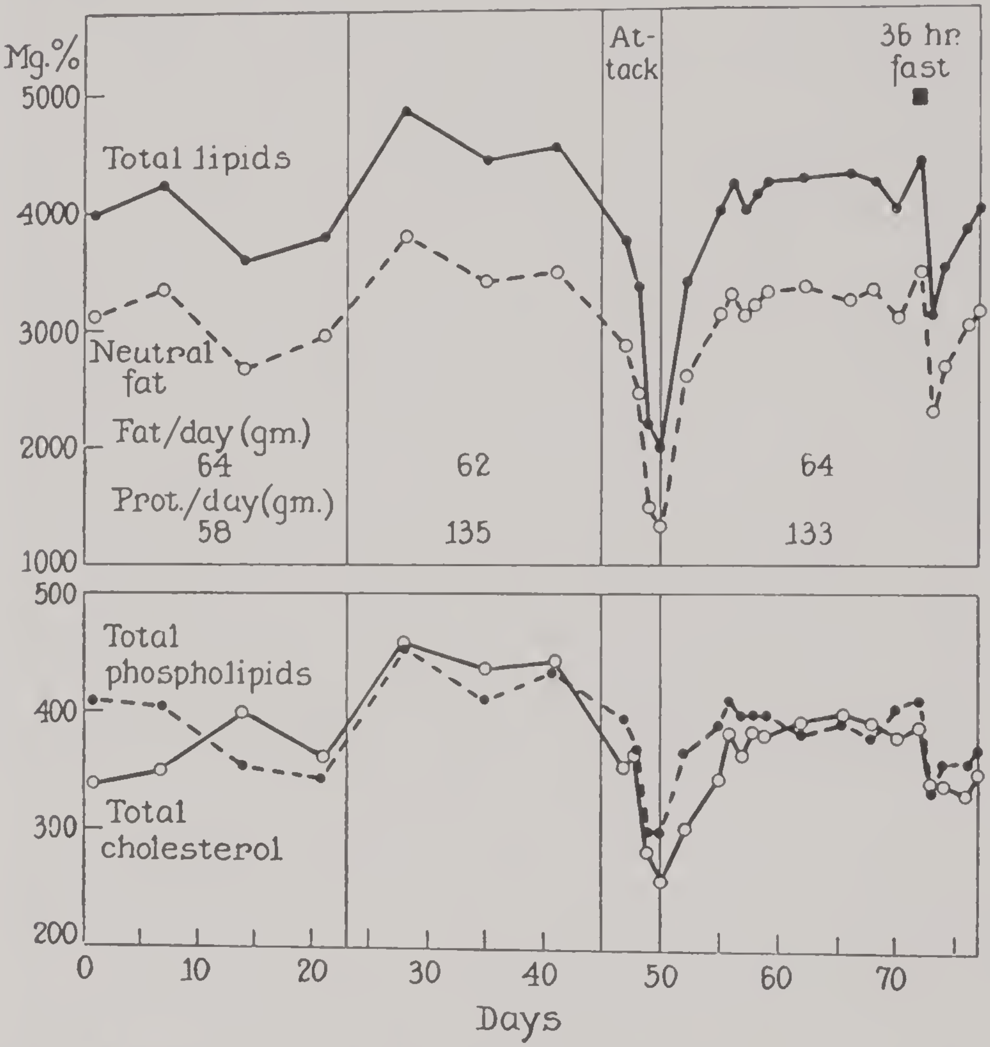

Figure 2. Serum lipid concentrations during same period of study as shown in Figure 1. All values on fasting sera. Ratio of free/total cholesterol ranged from 40–45%.

and from 23 to 46 days she consumed 135 ± 9.6 gms. protein per day. Although her caloric intake varied considerably (1648 ± 184), her body weight did not change significantly throughout the study (44.8 ± 0.68 k.). Throughout these

45 days her serum lipids were fairly constant. The serum was always grossly lipemic, and the lipid pattern was typical of essential hyperlipemia, with about 75 per cent of the lipid present as neutral fat (triglycerides) and with less striking elevations of cholesterol and phospholipids. The ratio of free-total cholesterol was consistently high, 40–45 per cent. Changing the protein intake from 63 to 135 gm. per day at the expense of carbohydrate, other elements remaining the same, had no apparent effect on the concentration of serum lipids.

On the forty-fifth day the patient complained of abdominal discomfort which gradually but relentlessly increased in severity. There had been no increase in serum lipids prior to this episode. By the forty-seventh day there was exquisite tenderness to deep palpation in the left hypochrondrium. Diaphragmatic excursion was small on both sides due to abdominal pain. However, board-like rigidity did not appear, and the liver and spleen were never palpable. Repeated flat plates of the abdomen showed no abnormalities including roentgen signs of ascites. Serum amylase and lipase levels were normal on several occasions prior to and during the attack. Although there was no indication of vascular collapse during the attack, a transfusion of 500 ml. of fresh blood was given after removing 500 ml. of the patient's blood, since Dr. Holt had reported a dramatic drop in serum lipids in this patient 19 years before when a blood transfusion was given. In the next 2 days the total serum lipids fell from 3740 to 2000 mg. per 100 ml. serum with the largest absolute and relative change in the neutral fat fraction. To test whether some factor in blood might have been responsible for this fall in serum lipids, various infusions were given to the patient in the weeks after the attack when she was asymptomatic. Fresh whole blood (500 ml.), fresh human plasma (250 ml.) and human serum albumin (25 gms.)

produced no apparent effect upon the serum lipid levels. It was concluded that the decrease in lipids during the attack was not significantly influenced by the transfusion given at that time.

Following the attack the patient was returned to her previous caloric, fat, and protein intake, except that all food was given in the form of formula feedings taken orally. Her formula was prepared daily by blending milk, milk protein (Lesofac, Wyeth), and chocolate syrup; a separate drink of another milk protein (Protinal, National Drug) also was given. All fat was thus of dairy origin. The constancy of food intake achieved by this maneuver proved so advantageous that complete formula feeding has been adopted at this hospital in many other metabolic experiments, although far simpler formulas have been devised. Complete formula feeding, while monotonous, has been otherwise acceptable to at least a dozen patients in whom it has been the sole source of food, and no harmful effects have been noted even after 4 months of continuous formula feeding. This can come as no surprise to a pediatric audience, although its acceptability in adult patients might not have been predicted.

On this dietary regimen the constancy of all lipid levels is indeed striking. The ordinarily highly labile neutral fat fraction varied only ± 7 per cent in 10 fasting sera in a two-week period, which is slightly more than the precision of the method for measuring this fraction. On the seventy-second day the patient was fasted for 36 hours with a striking decrease of all lipid levels. The largest absolute and relative decrease occurred in the neutral fat fraction. In view of this effect of a short period of fasting it was concluded that the decrease in lipids during her attack had been caused in part at least by the marked reduction in food intake during the attack.

Fifty mg. of intravenous heparin was followed within $1\frac{1}{4}$

hours by a fall in total serum lipids from 4603 to 3942 mg. per 100 ml. serum. It is of interest that all fractions fell to the same degree. The percentage decrease of the various fractions at the time of maximum change (1¼ hours) was neutral fat 15 per cent, free cholesterol 17 per cent, esterified cholesterol 15 per cent, and phospholipids 16 per cent. All fractions returned to pre-heparin levels 48 hours later. Sera diluted thirty times with saline showed a significant decrease in turbidity paralleling the fall in lipids, but the change was too small to be noted in the undiluted sera. Sera drawn at various intervals after heparin administration and allowed to stand undisturbed at 4°C. showed interesting differences in degree of separation of " cream " from subnatant serum. Pre-heparin sera and those drawn 5 hours or more after heparin all separated completely, with a sharply defined cream layer and a perfectly clear subnatant layer of serum. Darkfield examination of the cream layers, even after 3 months of storage, showed multitudes of discrete lipid droplets in Brownian movement. In contrast, sera drawn 15 minutes, 1¼ and 3¼ hours after heparin showed incomplete separation of " cream " from serum with no clear boundary between the two layers, and there was also a small whitish sediment in these tubes. Darkfield examination showed distinct clumping of the chylomicrons in the upper portion of the tubes, and in addition a number of typical fatty acid crystals were found in the sediment at the base. The appearance of the tubes is seen in Figure 3.

In summary, this twenty-nine-year-old female patient with familial hyperlipemia has had clinical evidence of her disease for 24 years, with the first appearance of tuberous xanthomata this year. There have been no signs of vascular involvement. Surgical exploration 3 years ago showed lacteal distension and chylous fluid in the abdomen. Present study showed grossly lipemic serum and elevation of all lipid fractions, with neutral fats making up 75 per cent of the lipids. When the diet was held constant, concentrations of the serum lipid fractions remained constant also. Intravenous heparin caused a temporary decrease in lipids and in turbidity. Transfusion of whole blood and infusion of plasma and of human serum albumin failed to affect serum lipid levels. Under our observation a typical abdominal crisis occurred. In contrast to the changes which Dr. Holt had seen repeatedly in this patient during childhood, there was no previous rise in serum lipids and no subsequent enlargement of liver or

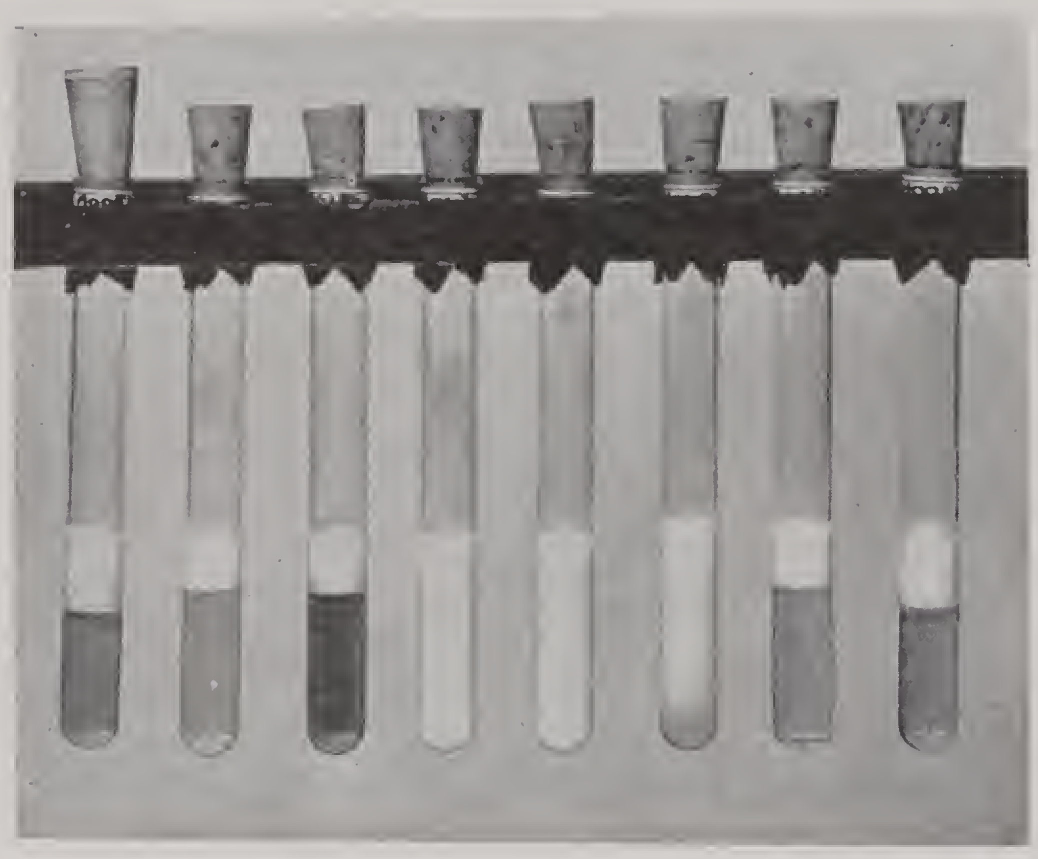

Figure 3. Sera stored without centrifugation at 4° C. Numbers 1–3 were sera drawn before administration of intravenous heparin (50 mg.), nos. 4–8 after heparin. No. 4—15 minutes, No. 5—1¼ hours, No. 6—3¼ hours, No. 7—5 hours, No. 8—21 hours after heparin.

spleen. Serum lipids decreased markedly in the course of this attack, but the patient's food intake also fell off in this period. There were no indications of chronic pancreatic insufficiency, nor of acute pancreatitis during this attack.

The following explanation of this patient's abdominal crises is postulated on the basis of her history and the present metabolic study. In this patient there may have been periodic temporary obstructions of the thoracic duct with chylous ascites and exudation of chyle into the retroperitoneal tissues and mesenteric root. Distension of these tissues would produce pain and occasionally vascular collapse. Anorexia and a markedly depressed food intake over a period of days would cause significant decreases in levels of serum lipids. Perhaps the thoracic duct became obstructed episodically by an intraluminal clumping of chylomicrons, or by clotting of its contents secondary to some effect of severe lipemia upon the clotting mechanism. This postulate is admittedly speculative.

5. Preparation, Utilization, and Importance of Neutral Fat Emulsion in Intravenous[1] Alimentation

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IN THE PRESENT REPORT I would like to discuss two closely related problems that have been studied in our laboratory during the past eight years. The problems are: (1) the preparation of fat emulsion suitable for intravenous administration to human patients; and (2) the importance of neutral fat (triglyceride) as a dietary constituent.

A. Preparation of Fat Emulsion for Intravenous Administration

It is convenient to discuss the preparation of fat emulsion first since in a number of cases we have employed these preparations in assessing the nutritive value of triglyceride fat.

The type and source of fats or oils that should be used in preparing fat emulsion was the first question considered. After extensive studies, it was decided that the " synthetic "

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[1] The work presented here was supported in part by a research grant from the Division of Research Grants, National Institutes of Health, U.S. Public Health Service, and in part by the Medical Research and Development Division, Office of the Surgeon General, Department of the Army, Contract No. DA-49-007-MD-252.

triglyceride[2] which is prepared by esterification of pure fatty acids with glycerol be used. This " synthetic " triglyceride preparation is composed of oleic acid, 60 per cent; palmitic acid, 30 per cent; stearic acid, 7.4 per cent; and linoleic acid, 2.6 per cent. The advantages in using the synthetic preparation are as follows: (1) It is made of pure materials and the impurities which are unavoidably present in the natural fats or oils can be eliminated. (2) Its fatty acid composition can be selected as desired. (3) Unlike the natural fats or oils, the processes for preparing the " synthetic " triglyceride can be controlled so that contamination with micro-organisms or their products may be avoided or greatly reduced.

Stabilizers and emulsifiers must be used in the preparation of a fat emulsion. From a great number of such agents which have been investigated, the following are employed: (1) Ethofat C/15, a fatty acid ester of polyethylene glycol; (2) Demal 14, a polyglycerol ester of oleic acid; (3) purified soybean phosphatides ; (4) Span 20, Sorbitan Monolaurate; and (5) sodium cholate. The composition of our currently used emulsion is shown in Table 1. Glucose is added to the emulsion to make it isotonic. Fat-soluble vitamins may also be incorporated in the emulsion if so desired and emulsions with higher fat concentration such as 20 or 30 per cent can also be prepared.

The apparatus used in preparing the emulsion is shown in Figure 1 and the procedures are as follows: The fat-soluble materials, Demal 14, Ethofat C/15, and Span 20, are dissolved in the oil and the water-soluble or -dispersible substances, such as glucose, sodium cholate, and soybean phosphatides are taken up in water. The oil-phase mixture is placed in the stirring tank and the water-phase mixture is added slowly with constant stirring. This mixture is then

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[2] Kindly supplied by the Oilseed Division, Southern Regional Research Laboratory, Department of Agriculture, New Orleans, Louisiana.

TABLE 1

COMPOSITION OF FAT EMULSION

Constituent	Amount (gm.)	Final Concentration (%)
Synthetic Fat	10.0	10.0
Demal–14	0.5	0.5
Ethofat C/15	0.5	0.5
Span 20	0.25	0.25
Purified Soybean Phosphatides	0.5	0.5
Sodium Cholate	0.1	0.1
Glucose	5.0	5.0
Dist. Water to	100.0 ml.	

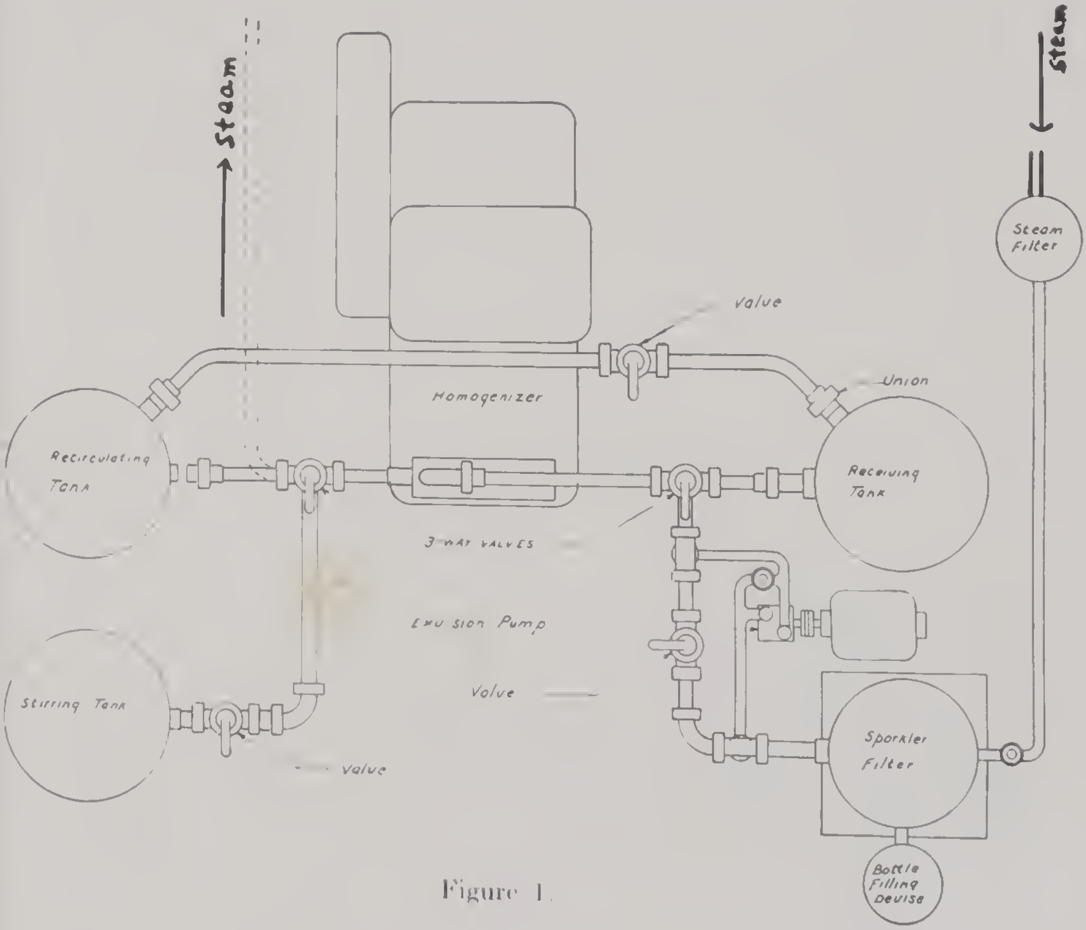

Figure 1.

directed into a dairy homogenizer and homogenized at pressure of approximately 4,000 pounds pressure per square inch. The homogenization is repeated five or six times in this apparatus by allowing the emulsion to cycle back and forth through the homogenizer which is placed between the receiving and recirculating tanks. No air is allowed to enter the homogenizer at any time during the entire process. After the last homogenization the emulsion is pumped through a Sparkler Filter and is bottled. The bottles, which are previously sterilized, are filled in a closed system which limits the exposure to air or possible contamination. The bottled emulsions are then autoclaved at 110° C. for 12 minutes. The properties of the emulsion are listed in Table 2.

TABLE 2

PROPERTIES OF FAT EMULSION

1. pH: 6.5–6.9
2. Particle size: 0.5–1.5μ average less than 0.5μ
3. Stability: 6 months or more
4. Antigenicity: non-antigenic
5. Sterility: negative both aerobically and anaerobically
6. Pyrogenicity: ?

B. The Importance of Neutral Fat (Triglyceride) as a Dietary Constituent

We would like to present evidence that an adequate level of triglyceride fat in the diet is essential for optimal growth or weight gain in rats, in dogs, and in human subjects.

Ever since the classical experiments of Evans, Burr and associates (1-8) demonstrating the essentiality of certain unsaturated fatty acids for rats, the importance of these substances in the diet has been recognized. Similar evidence was obtained by other investigators (9-12) using puppies and mice. It has also been demonstrated by Forbes, Swift and coworkers (13-17) and Deuel, Scheer, and associates (18-22) that rats fed diets with high fat levels showed a more rapid weight gain, greater nitrogen retention, increased physical capacity for work, increased resistance to starvation, etc. than rats on fat-free or low fat regimens. Deuel, *et al.* (22) first suggested that the beneficial effect of fat in the diet cannot be explained entirely on the basis of essential fatty acids. They showed that when methyl linoleate was given as a supplement to rats on a fat-free diet, a prompt response in growth resulted. However, the weight curve of these rats was soon plateaued, but feeding of a diet containing 10 per cent cottonseed oil to these rats resulted in a further acceleration of gain in weight.

1. Nutritive Value of a "Synthetic" Triglyceride Preparation Fed by Mouth to Weanling Rats.

This work was to determine whether or not the synthetic triglyceride with known fatty acid composition can be utilized by the weanling rats as well as other natural edible fats or oils. Weanling rats weighing from forty to forty-five grams were divided into groups of six or twelve each. The individual groups were fed the following diets: (1) fat-free, (2) five or 25 per cent "synthetic" triglyceride, and (3) five or 25 per cent lard (controls). Food and water were allowed *ad libitum.* The experiment was carried out for 13 weeks.

There are two things demonstrated in Figure 2: (1) rats fed the fat-free diet failed to gain as much weight as those on diets containing lard or "synthetic" triglyceride; (2) there was no difference in the growth curves between the 25 per cent lard and 25 per cent "synthetic" triglyceride groups. In addition to the retarded growth of rats fed the fat-free diet, they developed pathologic changes in the hair and skin characteristic of essential fatty acid deficiency. No

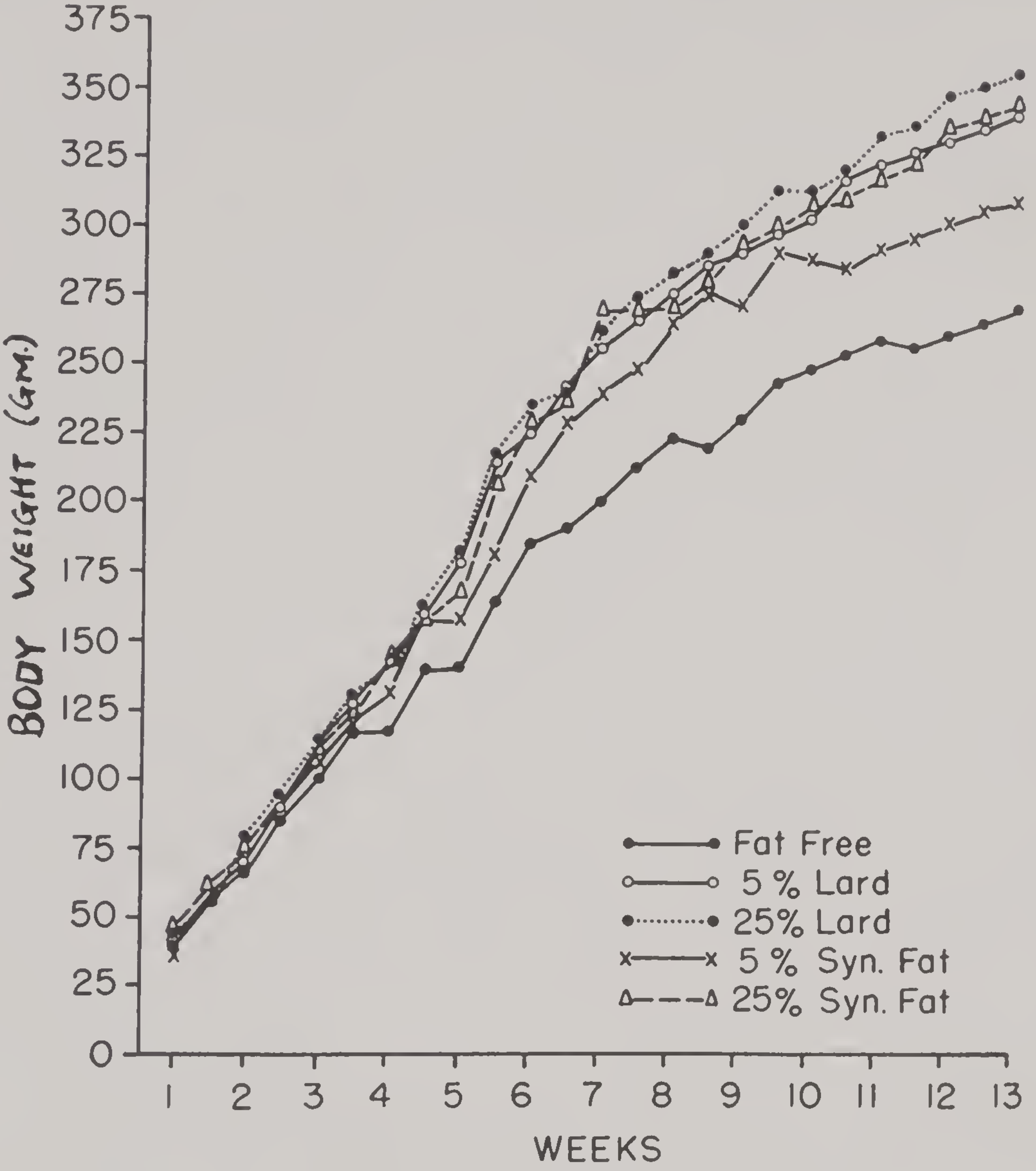

Figure 2.

such findings were observed in rats fed lard or "synthetic" triglyceride diets.

It can be seen in Table 3 that the average daily gain in body weight per 100 gms. rat in the fat-free group was 2.5 gms., which is 17 to 30 per cent lower than the weight gain of the groups receiving fat in the diet. However, the caloric intake of the rats on the fat-free diet was 37.1 per 100 gms. rat per day, which is slightly higher than the other groups. The lowest caloric intake was seen in the 5 per cent "synthetic" triglyceride group and this probably explains why the weight gain of this group was slightly less than that of the other groups receiving fat. The average caloric intake (per 100 gms. rat per day) required per gram of daily weight gain is 14.6 for the fat-free group. The value is 10.7, 10.3, 11.3 and 10.8 for 5 per cent lard, 25 per cent lard, 5 per cent "synthetic" triglyceride and 25 per cent "synthetic" triglyceride groups respectively.

TABLE 3

AVERAGE CALORIC INTAKE AND DAILY GAIN IN BODY WEIGHT

Diet	Caloric intake /100 gm. rat/day				Average daily gain in body wt./rat (gm.)
	Total	from fat	from protein	from carbohydrate	
Fat-free	37.05	—	12.22	24.84	2.54
5% Lard	36.13	4.20	11.20	20.73	3.39
25% Lard	36.69	16.94	8.97	10.79	3.56
5% Synthetic Fat	33.95	3.87	10.55	19.27	3.00
25% Synthetic Fat	36.58	16.93	8.90	10.73	3.39

The experiment demonstrates the importance of fat in the diet for optimal growth. Fat in the diet apparently favors a more efficient utilization of calories since the rats on the fat-free diet showed the poorest growth despite the highest caloric intake. It may be further noted that the " synthetic " triglyceride was apparently equivalent to the natural fat as a dietary constituent.

2. Nutritive Value of " Synthetic " Triglyceride Emulsion Administered Intravenously to Dogs.

The experiment was carried out as follows: Three healthy adult dogs were fed a normal basal diet furnishing eighty calories per kg. of body weight per day, of which 50 per cent came from carbohydrate, 16 per cent from protein and 34 per cent from fat as lard for 3 weeks (Period I). Immediately following this period they were given a fat-free diet by mouth plus 3.0 gms. of fat per kg. of body weight per day as a 10 per cent " synthetic " triglyceride emulsion by vein (Period II). The regime did not alter the total caloric intake or proportions of carbohydrate, protein, fat, vitamins and minerals given during Period I except that the lard in Period I was replaced by " synthetic " triglyceride in Period II. The animals were maintained on this regime for a period of 4 weeks, after which the injection of " synthetic " triglyceride emulsion was discontinued and they were placed on a fat-free diet (Period III). Figure 3 shows the average changes in body weight, caloric intake and nitrogen balance of the three dogs during the three periods. It can be seen that the average gain in weight was 0.4 kg. or 3 per cent of the initial weight during Period I. The average gain in weight was 0.6 kg. or 5 per cent of the body weight during Period II. During Period III, however, the dogs lost 0.8 kg. or 6 per cent of the body weight. The nitrogen retention during Periods I and II was of about the same magnitude but was much reduced during Period III. The average daily nitrogen balance of the three dogs was + 2.6 and + 2.1 gms. for

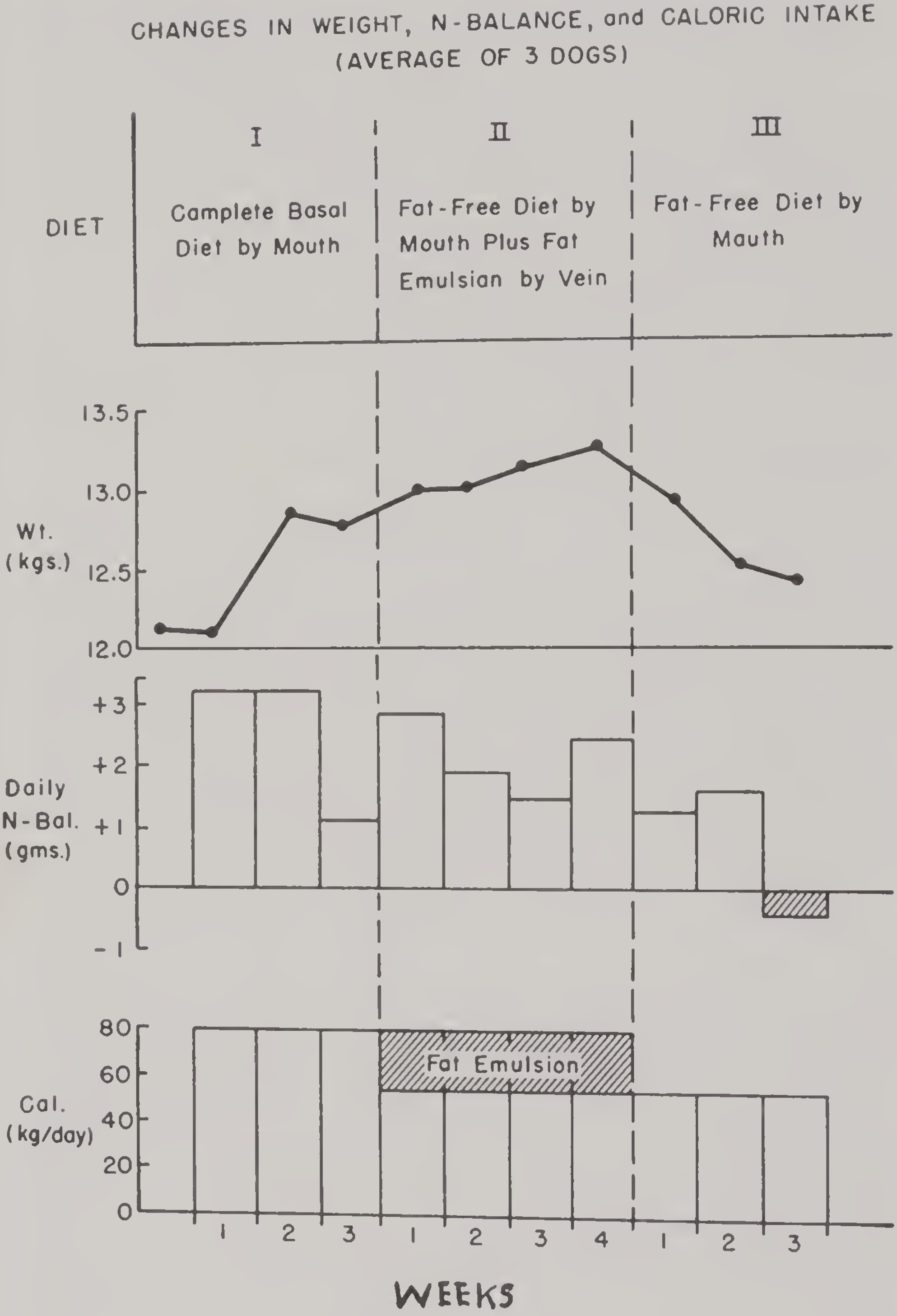
CHANGES IN WEIGHT, N-BALANCE, and CALORIC INTAKE
(AVERAGE OF 3 DOGS)
DIET
I
Complete Basal Diet by Mouth
II
Fat-Free Diet by Mouth Plus Fat Emulsion by Vein
III
Fat-Free Diet by Mouth
Wt. (kgs.)
13.5
13.0
12.5
12.0
Daily N-Bal. (gms.)
+3
+2
+1
0
-1
Cal. (kg/day)
80
60
40
20
0
Fat Emulsion
1
2
3
1
2
3
4
1
2
3
WEEKS

Figure 3.

Periods I and II respectively, but it was reduced to + 0.9 gm. during Period III.

No significant changes were noted in the BSP clearance, serum alkaline phosphatase, plasma albumin, globulin and plasma NPN. Glycosuria and albuminuria were never observed during the entire course of experiment. No untoward reactions were observed during the period of intravenous administration of synthetic triglyceride emulsion.

The results indicate that (1) synthetic triglyceride given intravenously as a fine emulsion is utilized and does not cause any apparent toxic reaction; (2) fat is a dietary factor for weight gain or growth in dogs as in rats.

3. Studies of Intravenous Alimentation in Dogs (23, 24).

In view of the high metabolic requirements of fever, trauma, wounds, surgical operations, and other disease conditions, it is very likely that the effects of fat deficiency are much more serious in sick individuals than in healthy ones. We therefore undertook experiments to determine the importance of neutral fat (triglyceride) in intravenous alimentation in dogs over a prolonged period of time.

Five healthy adult dogs were used. In the control period they were fed a normal basal diet by mouth furnishing eighty calories per kg. of body weight per day as described previously. Immediately following this period, four animals (Dogs B, R, C, and M) were given a diet containing the same amount of carbohydrate, protein, and fat, but administered exclusively by vein. The carbohydrate was in the form of glucose, the protein as a casein hydrolysate (Amigen), and fat as a 10 per cent olive oil emulsion. Minerals and vitamins were also supplied in adequate amounts. Water was allowed *ad libitum*. The fifth dog (Dog Y) received the same amount of carbohydrate, protein, minerals, and vitamins but without the neutral fat. The caloric intake of

Dog Y during the intravenous feeding period was 53 calories per kg. of body weight per day which was inadequate.

All animals (Dogs B, R, C, and M) which received complete intravenous alimentation including the neutral fat in the form of olive oil emulsion remained healthy and lively throughout. Their hair was smooth and shining. However, Dog Y, which received no neutral fat, appeared emaciated and apathetic. Its hair was very coarse and dry, and epilation was starting to occur on the hind legs. The appearance of these animals can be compared in Figures 4 and 5.

Figure 6 shows the changes in body weight and nitrogen balance of the dogs during the course of the experiment. The animals receiving complete intravenous alimentation including neutral fat maintained or slightly increased their body weight during the period of infusion. However, Dog Y, which did not receive fat, lost 14 per cent of its initial weight. The nitrogen balance of the four dogs receiving fat was slightly variable during the period of infusion. A negative balance was observed during one or another week during both the control and the infusion periods in all the animals except Dog C. The nitrogen retention of Dog Y during infusion was less than that during the control period.

A number of tests which included rose bengal clearance, serum alkaline phosphatase, total plasma proteins and plasma NPN, R.B.C. and W.B.C. count, blood hemoglobin, hematocrit and water balance did not reveal any abnormalities attributable to the procedure. The H and E and Sudan IV stained histologic sections of liver, lung, spleen, kidney, heart, aorta, pancreas, stomach, intestine, adrenal and thyroid glands, and testis or ovary did not show any significant changes. The results demonstrate that (1) complete intravenous alimentation with protein, carbohydrate, and fat for prolonged periods is feasible and compatible with excellent

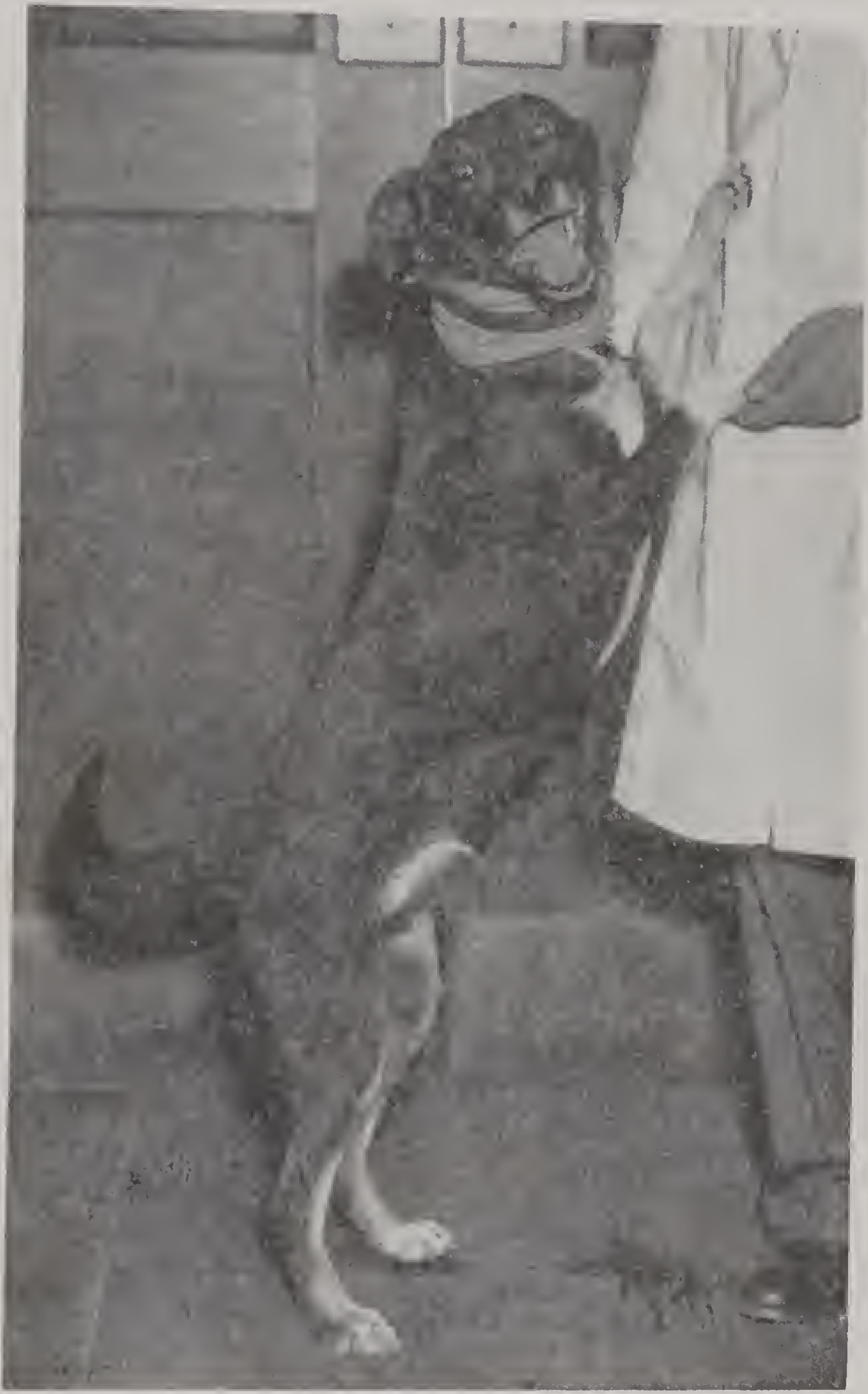

Figure 4.

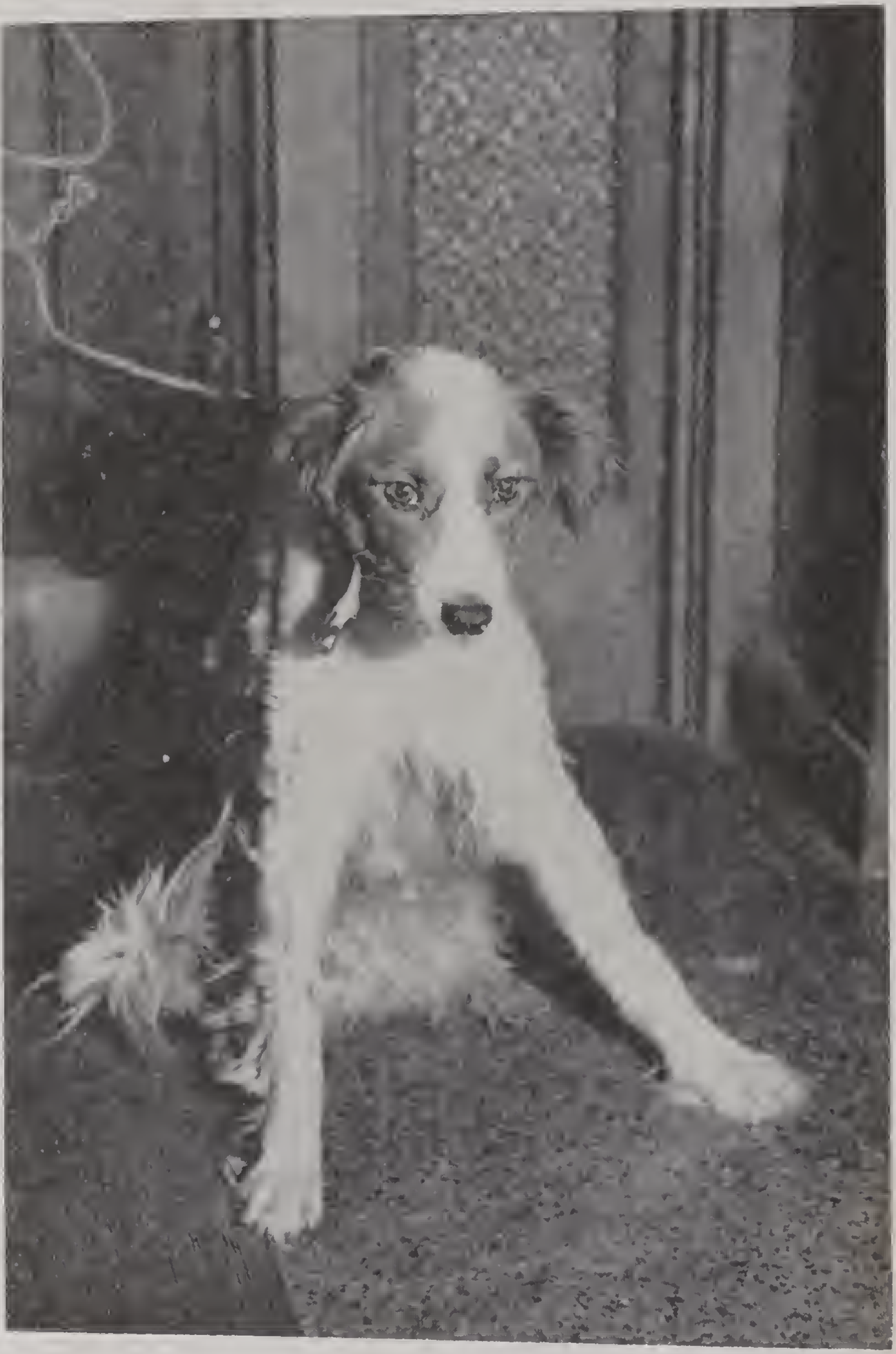

Figure 5.

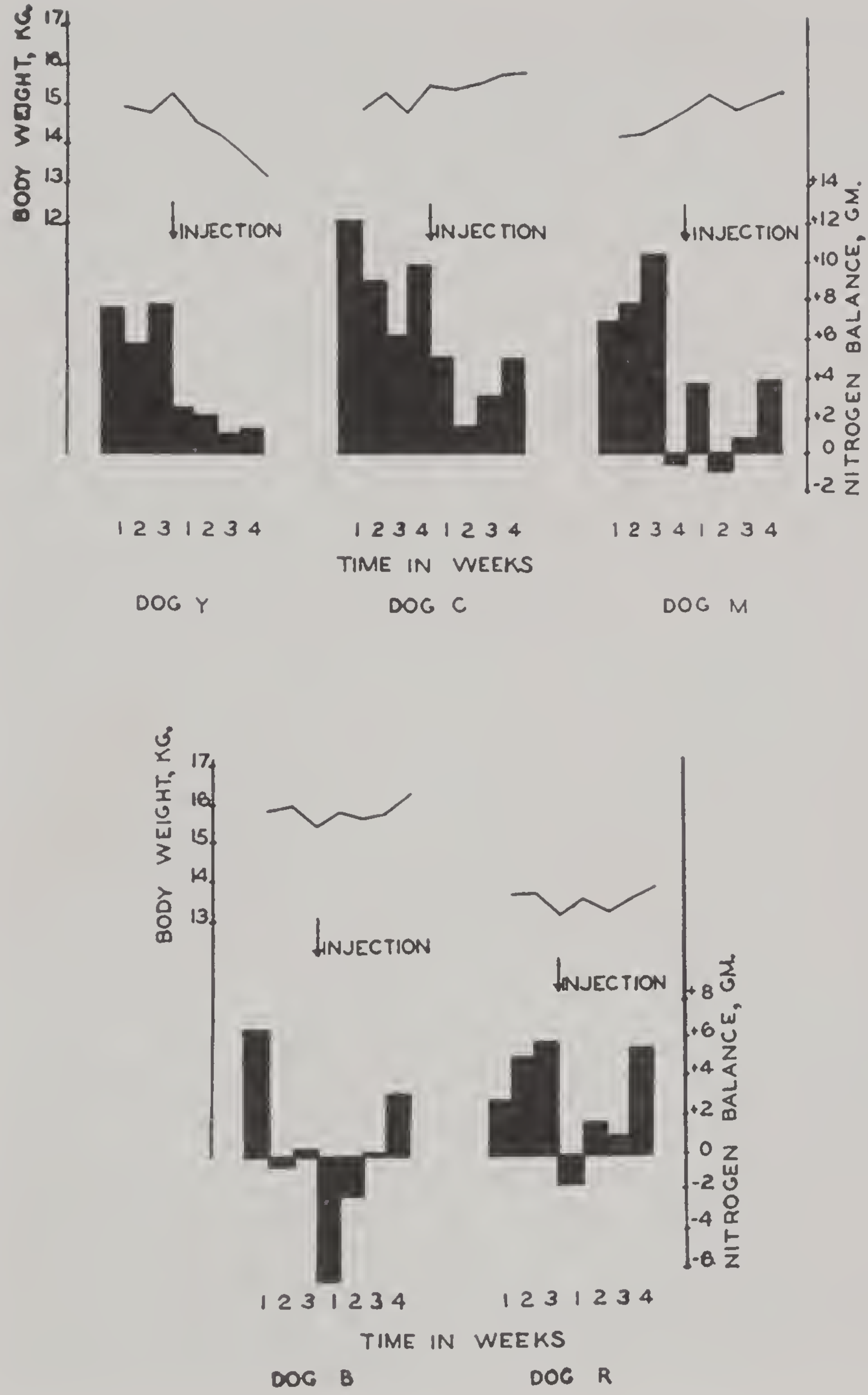
BODY WEIGHT, KG.
17
16
15
14
13
12
INJECTION
INJECTION
INJECTION
NITROGEN BALANCE, GM.
+14
+12
+10
+8
+6
+4
+2
0
-2
1 2 3 1 2 3 4
1 2 3 4 1 2 3 4
1 2 3 4 1 2 3 4
TIME IN WEEKS
DOG Y
DOG C
DOG M
BODY WEIGHT, KG.
17
16
15
14
13
INJECTION
INJECTION
NITROGEN BALANCE, GM.
+8
+6
+4
+2
0
-2
-4
-6
1 2 3 1 2 3 4
1 2 3 1 2 3 4
TIME IN WEEKS
DOG B
DOG R

Figure 6.

health; (2) utilization of intravenously administered fat emulsion is confirmed.

However, the loss of weight in Dog Y, which did not receive fat in the above experiment, may have been due to (1) insufficient caloric intake, (2) a deficiency of essential fatty acids, (3) a deficiency of triglyceride fat, or (4) a combination of these factors. In the next experiment we undertook to investigate the cause of weight loss, or more specifically, whether a diet furnishing adequate calories and essential fatty acids but deficient in neutral fat would maintain health and growth.

The experiment was carried out as follows: In the three-week control period, five healthy adult male dogs were fed a normal basal diet furnishing eighty calories per kg. of body weight per day as in the previous experiments. Immediately following this period, one dog (T) which was the control animal received the same caloric intake of protein, carbohydrate, and fat, but administered by vein with the same constituents as in the previous experiment. Two dogs (Br and D) also received the intravenous feeding, except that glucose was substituted isocalorically for fat and a supplementation of essential fatty acids was given. Methyl linoleate, methyl linolenate and methyl arachidonate in the amounts of 100 mg., 100 mg., and 10 mg. per kg. of body weight per day respectively were given by mouth. The other two dogs (S and J) received isocaloric intravenous alimentation with no fat or essential fatty acid supplementation. All five dogs received the same amount of protein, vitamins, and minerals. The period of intravenous alimentation again lasted for four weeks.

The control Dog T remained healthy and lively throughout. Dogs Br and D, which received only the essential fatty acid supplement, were not as lively and alert as Dog T, but no abnormal changes in their hair were observed. Dogs S and J, which received neither fat nor essential fatty acids, appeared apathetic and emaciated. Their hair was coarse, dry, and fell out rapidly. These conditions can be seen in Figures 7, 8, and 9 which are photographs of Dogs T, Br, and S respectively, taken at the end of the four-week period of intravenous alimentation.

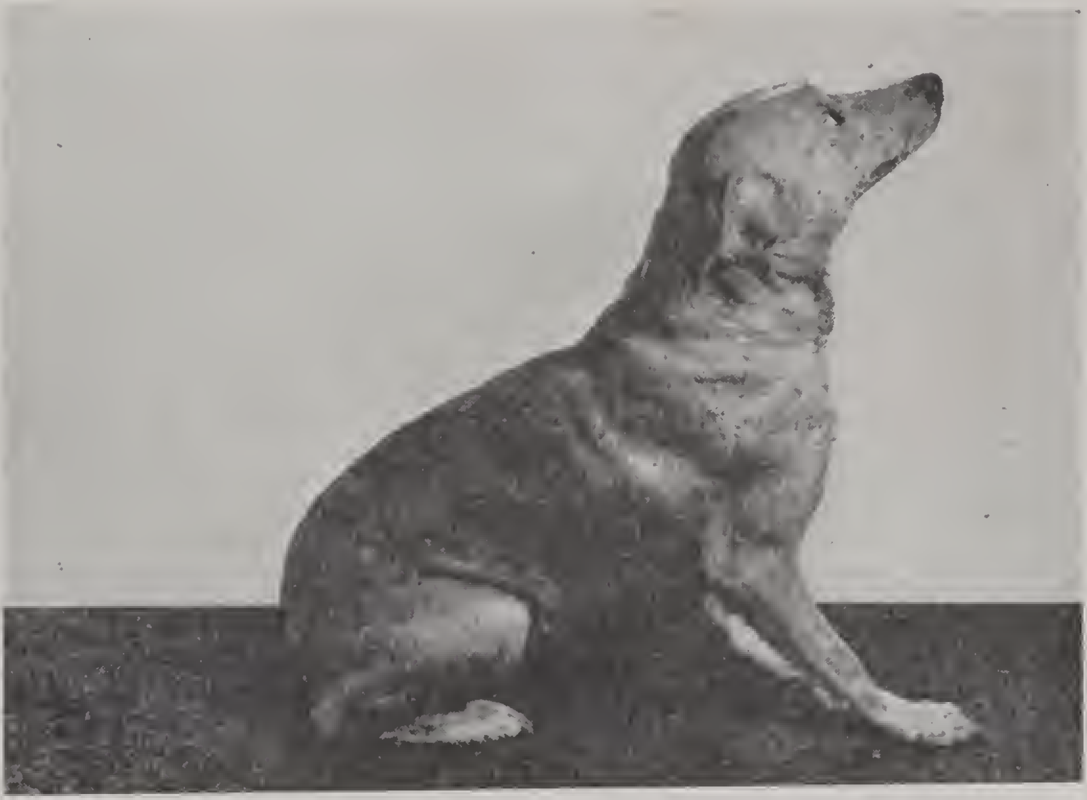

Figure 7.

The changes in body weight and nitrogen balance of the dogs during the course of experiment are shown in Figure 10. It can be seen that the control animal on complete intravenous feeding with fat gained 7.6 per cent of its initial weight during the four-week period. Dogs Br, D, S, and J, on the other hand receiving the same total caloric intake but lacking fat, lost 9.2, 4.3, 9.1, and 6.2 per cent respectively of their initial weights. Supplementation of essential fatty acids to Dogs Br and D did not prevent weight loss. In no

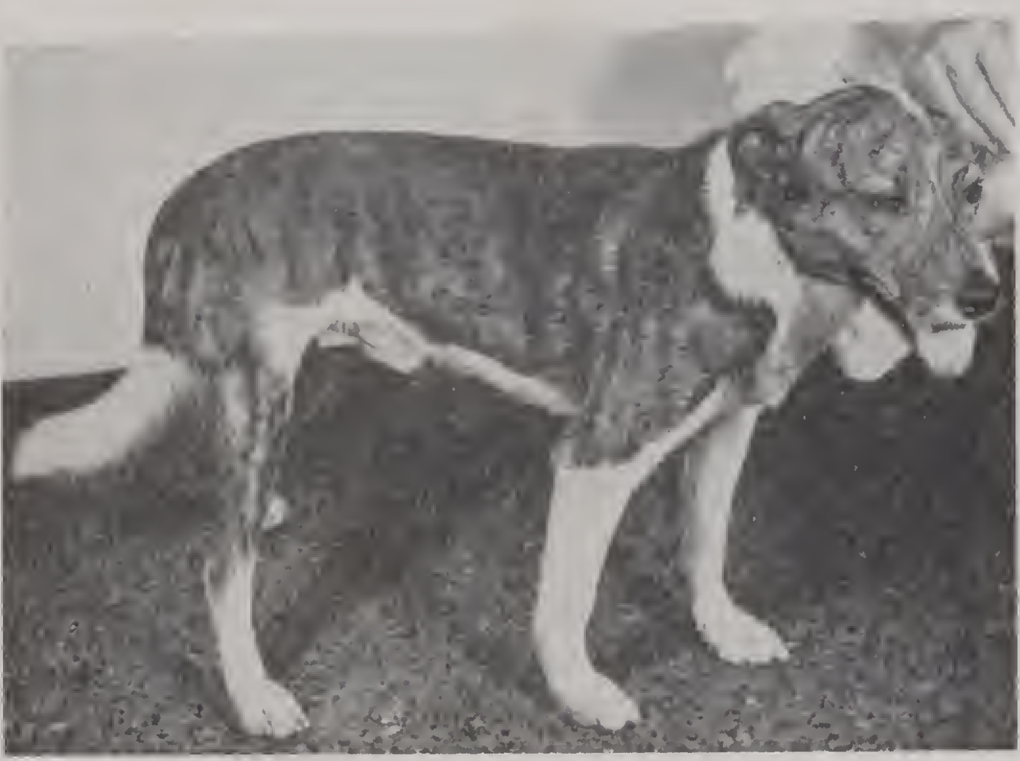

Figure 8.

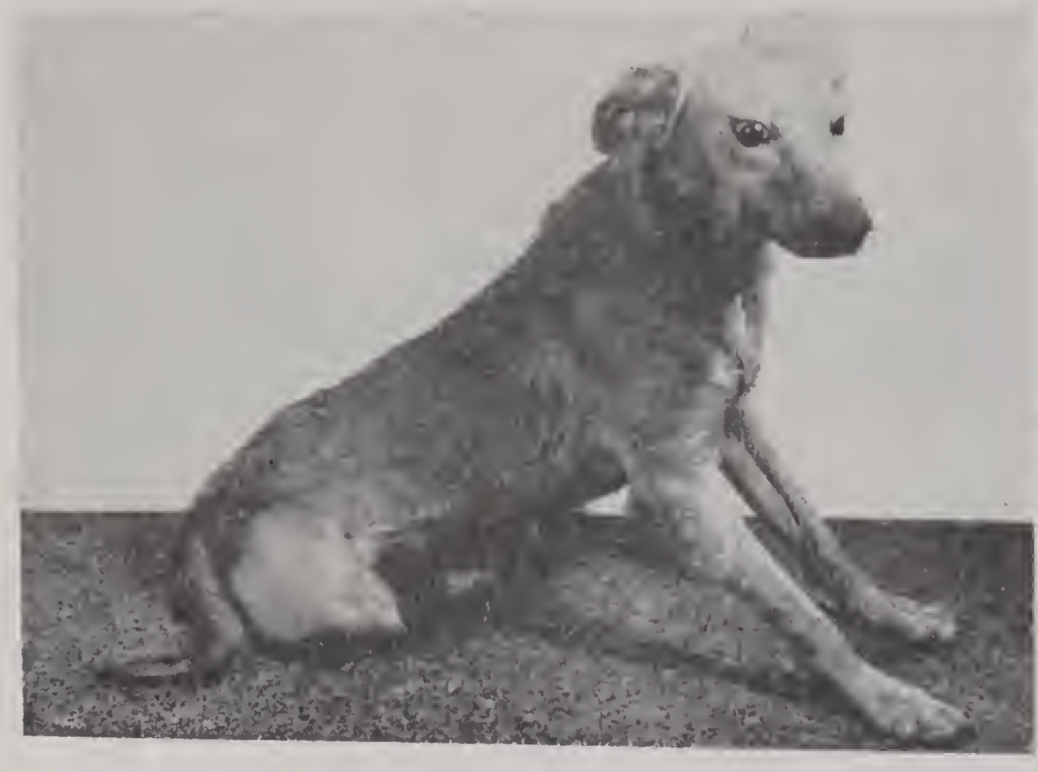

Figure 9.

instances was the nitrogen balance negative in the control animal although nitrogen retention was considerably less

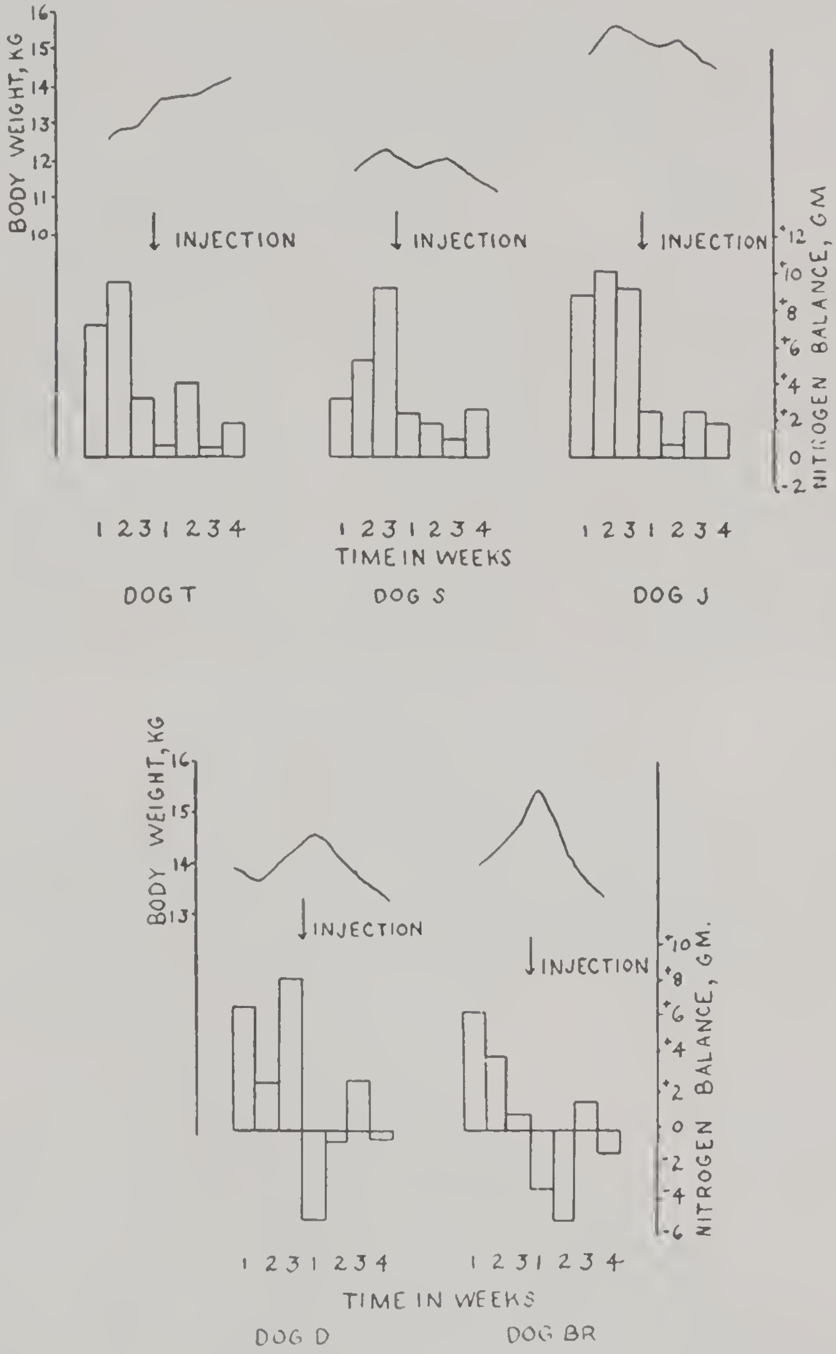

Figure 10.

during intravenous alimentation than in the control period. However, Dogs Br and D, the animals which received essential fatty acids, demonstrated negative balance during the first, second, and fourth weeks of injection. Dogs S and J were not different from the control animal.

As in the previous experiment, the tests described earlier were carried out and a histologic study was also made following sacrifice. No significant abnormalities could be attributed to the treatment.

These results indicate that (1) without neutral fat, an adequate diet with respect to calories, protein, the known vitamins and minerals does not promote body weight gain. The administration of essential fatty acids prevents certain specific pathologic changes but does not permit optimal growth and well-being in the absence of neutral fat; (2) intravenous infusion of a complete diet containing adequate triglyceride fat is compatible with excellent health and body weight gain.

4. The Use of Neutral Fat Emulsion in Intravenous Alimentation in Human Patients.

The value of neutral fat in nutrition and the finding that intravenously administered fat is well utilized and non-toxic in animals made it desirable to test the use of fat emulsion by vein in selected patients. More than two hundred patients have received intravenous fat emulsion in amounts of 500 to 1,000 ml. per day for from 1 to 30 days. Two cases are presented here to illustrate typical observations.

Case 1. Patient F. P., a white male, age 42. The diagnosis of carcinoma of the splenic flexure with gastrointestinal obstruction was made after exploratory laparotomy. Colostomy was also performed for the purpose of feeding. However, it was found that feeding through colostomy was unsatisfactory and inadequate. The patient's condition was going downhill and weight loss was rapid. He was given intravenous injection of protein hydrolysate, glucose solution, vitamins, and minerals to supplement his inadequate food intake through colostomy. The total caloric intake was from 1900 to 2300. In spite of this intake, the patient did not improve significantly and body weight remained at a stationary and low level, although the nitrogen balance was slightly positive. One liter of a 10 per cent olive oil emulsion with 5 per cent glucose furnishing 1,100 calories was then injected intravenously daily for 7 days. It can be seen in Figure 11 that beginning from the second day of fat administration, his body weight began to increase and after 7 days on this regime, the patient had gained 8 pounds. Furthermore, the nitrogen balance during the period of fat administration became more strongly positive.

Case 2. Patient I. B., a white male, age 69. A diagnosis of benign prostatic hypertrophy and probable carcinoma of the stomach was made and a transurethral prostatic resection and vasectomy were performed. A rapid weight loss and very poor appetite made it advisable to supply nutrition parenterally. The following study was carried out. During the first three-day control period, patient was given protein hydrolysate, invert sugar solution, vitamins and minerals furnishing 1600 calories per day. During the next five-day period, fat emulsion was given in addition to the nutrients injected during the three-day control period. The amount of calories supplied by fat was from 360 to 900 daily with a total daily intake of from 1500 to 2500. The results of this study are shown in Figure 12. It can be seen that the patient gained weight during the five-day period of intravenous alimentation with fat added. The nitrogen retention and potassium balance were also more positive during the infusion period with fat. The average water balance was less in the period of fat infusion than that in the control period. Intravenous administration of fat did not produce

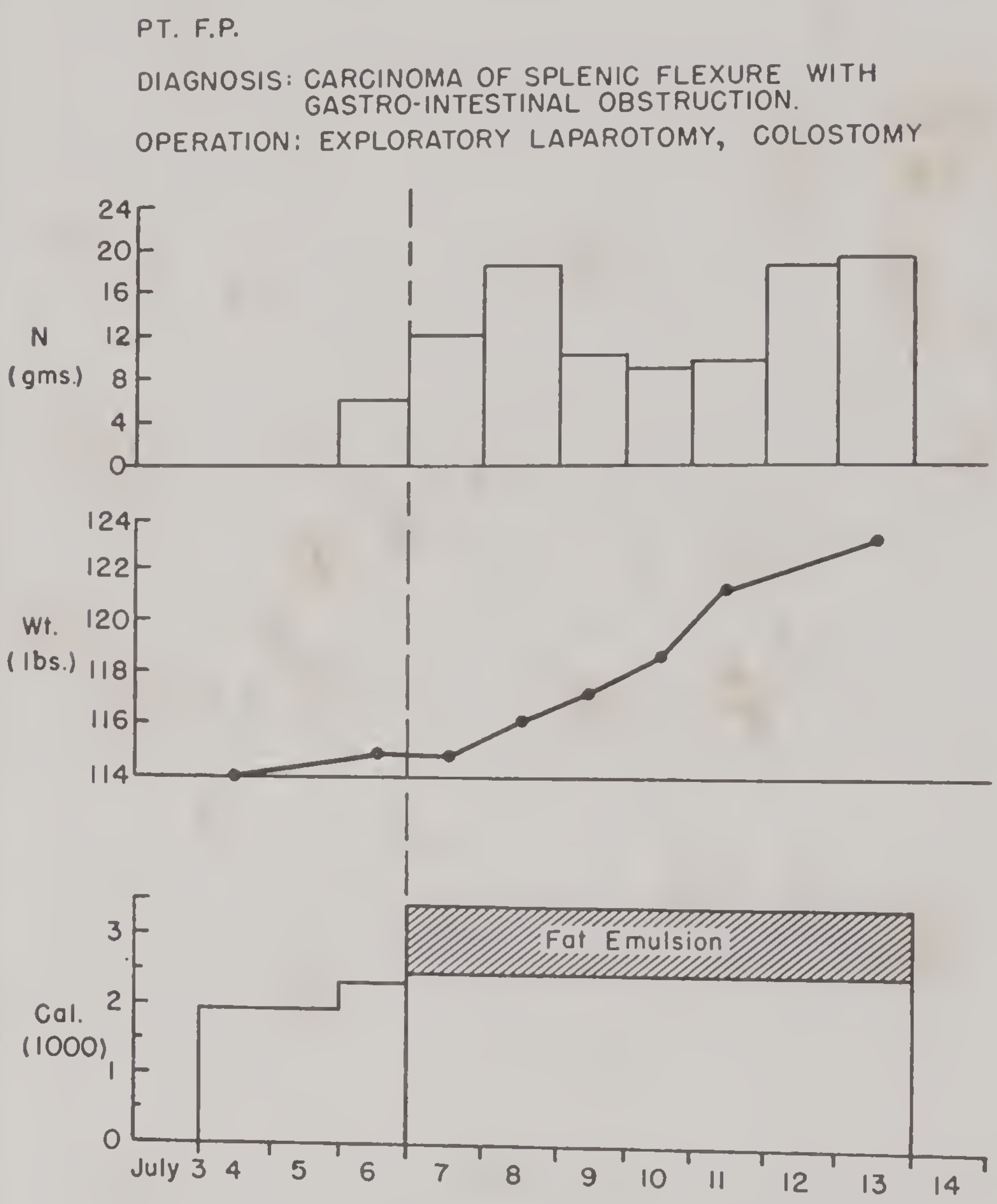

Figure 11.

febrile reaction. In fact, the body temperature during fat injection was lower than that in the control period.

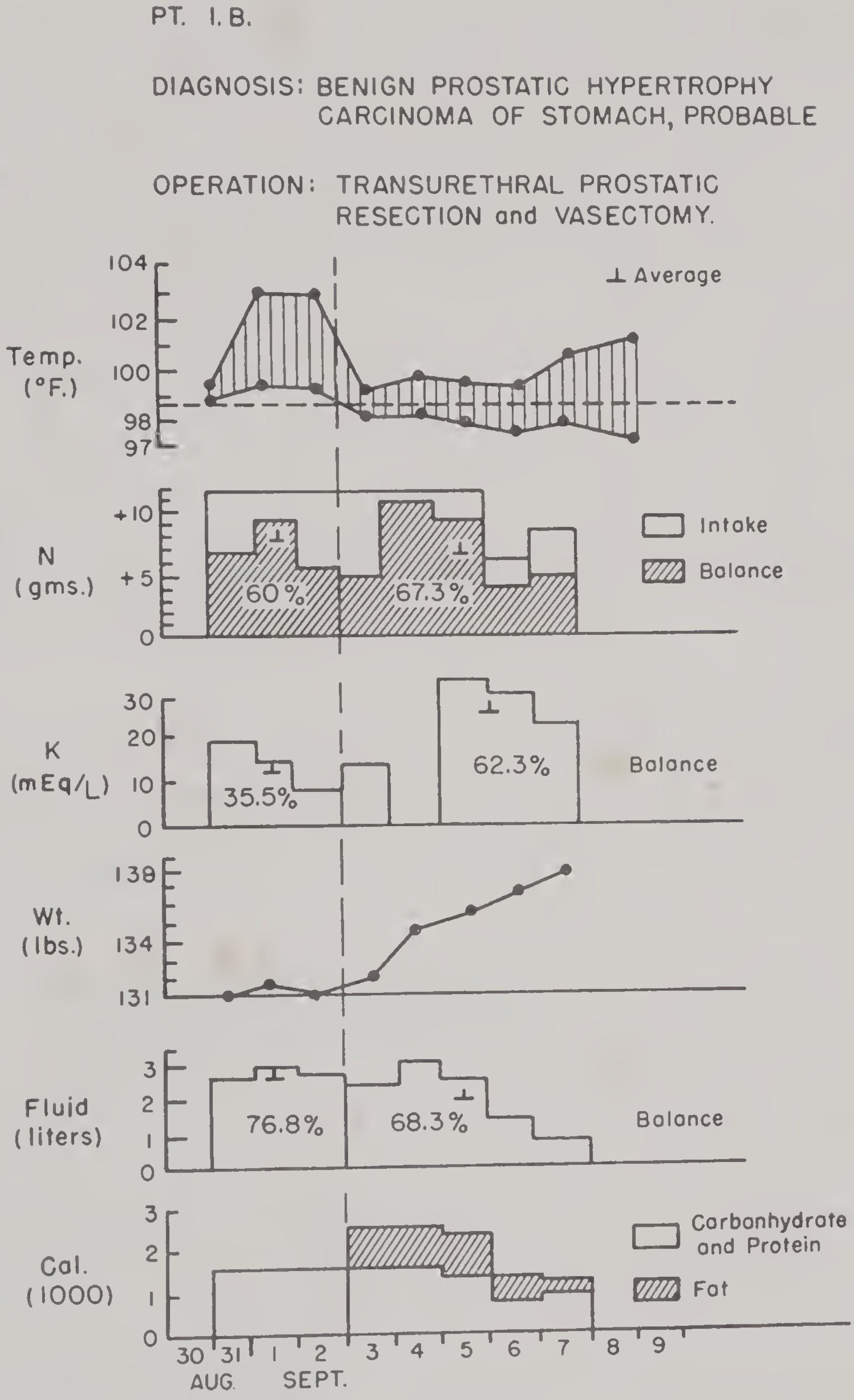
PT. I.B.
DIAGNOSIS: BENIGN PROSTATIC HYPERTROPHY
CARCINOMA OF STOMACH, PROBABLE
OPERATION: TRANSURETHRAL PROSTATIC
RESECTION and VASECTOMY.
Temp. (°F.)
104
102
100
98
97
⊥ Average
N (gms.)
+10
+5
0
60%
67.3%
Intake
Balance
K (mEq/L)
30
20
10
0
35.5%
62.3%
Balance
Wt. (lbs.)
138
134
131
Fluid (liters)
3
2
1
0
76.8%
68.3%
Balance
Cal. (1000)
3
2
1
0
Carbonhydrate and Protein
Fat
30
31
1
2
3
4
5
6
7
8
9
AUG.
SEPT.

Figure 12.

SUMMARY AND CONCLUSION

(1) The preparation of a fine, stable, and non-toxic neutral fat emulsion suitable for intravenous administration is described.

(2) This emulsion administered intravenously to dogs and human subjects is biologically utilized and does not cause any apparent toxic reactions.

(3) The importance of neutral fat in the diet for optimal growth or weight gain is demonstrated.

a. The administration of adequate calories, protein, the known vitamins and minerals, but without neutral fat, does not promote optimal body weight gain and well-being. The administration of essential fatty acids prevents certain specific pathologic changes but does not permit optimal growth and well-being in the absence of neutral fat.

b. Complete parenteral alimentation with protein, carbohydrate, fat, vitamins and minerals has been carried out for prolonged periods and is compatible with weight gain and excellent health.

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6. On the Role of Lipemia Clearing Factor in Lipid Transport

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THE STATE OF LIPIDS in plasma and tissues has been the subject of considerable study during recent years.[1] These studies have shown that lipids exist virtually exclusively in the form of lipoprotein complexes. Our understanding of the phenomenon of lipid transport and metabolism must, therefore, be directed, at least in part, toward an examination of the enzymatic and hormonal control of lipoprotein synthesis and transformation. In addition, it is clear that the study of aberrations in lipid metabolism occurring in various metabolic diseases must similarly involve such considerations.

The chemistry and physical description of the various lipoproteins of plasma in health and disease has been reviewed extensively of late (1-8). I will not, therefore, attempt to cover this comparative material in detail at the present time, particularly as regards specific disease entities. Instead, I would like to concentrate on the more general aspects of lipoprotein metabolism in plasma.

Lipoproteins form a class of compounds with varying densities and containing different proportions of protein, phospholipid, cholesterol, and triglycerides. These proteins may be studied conveniently by a variety of techniques, including ultracentrifugal, electrophoretic and chemical methods. Techniques introduced by Gofman and his collaborators (6), Gurd and Oncley (9), and Pedersen (10) permit the separation of the various density classes by means of flotation at high centrifugal speeds under chosen conditions of solvent density. At a density of 1.063, for example, the β lipoprotein class as well as lipoproteins of uncertain composition of even lower density, can be separated from the α lipoprotein components. Similar separations are possible by electrophoresis on starch (8) or by ethanol fractionation (7).

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[1] The various original studies from the National Heart Institute described in connection with the following discussion were carried out by Drs. E. Boyle, J. Bragdon, R. Brown, R. Gordon, R. Havel, E. Korn, and C. Anfinsen.

In the majority of diseases involving abnormal lipid metabolism, the β lipoprotein fraction of plasma is considerably increased in concentration, whereas the α lipoproteins appear to decrease. The correlations earlier observed between the levels of plasma cholesterol and the incidence of atherosclerosis or of such diseases as diabetes and myxedema are now interpretable on the basis of the fact that the low density lipoproteins constitute the major vehicle for cholesterol transport in plasma. It would appear a fruitful approach to the study of such diseases, therefore, to examine the mechanisms by which the normal pattern of plasma lipoproteins is maintained.

Immunological studies have indicated that the normal β_1 lipoprotein is strongly cross-antigenic with lipoproteins of lower density and higher S_f * (11). In addition, it has been frequently demonstrated that these latter molecules are rapidly degraded in the normal individual, following ingestion of fat (4), in the direction of the normal β lipoprotein component, passing successively through stages of higher

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* The symbol S_f indicates Svedberg units of flotation under the conditions described by Gofman et al. (6).

density and lower triglyceride content during their metabolism. One explanation for this progressive change is the possibility that the very low-density lipoproteins may simply constitute normal β_1 lipoprotein molecules containing varying amounts of triglyceride in their basic matrix.

Heparin induced clearing factor. During the past two years our group has concerned itself with the study of an enzymatic mechanism stimulated by heparin administration which may play an important part in the normal degradation of the transient low-density lipoproteins. In 1943, Hahn (12) observed that the injection of heparin caused the rapid disappearance of chylomicrons from the post-absorptive plasma of dogs. These studies have been greatly extended in numerous laboratories (13–21) and the references quoted at the end of this discussion list many of the observations that have been made since Hahn's original discovery.

When heparin is injected intravenously into humans or experimental animals whose plasmas contain elevated levels of chylomicrons or of abnormal low-density β lipoproteins, these components rapidly disappear from the plasma. It has also been possible to produce the clearing factor responsible for these changes *in vitro* as well as *in vivo* (16, 22). Thus, by the incubation of normal plasma, containing no detectable clearing factor activity, with heparin in the presence of extracts from various tissues, one can demonstrate the production of triglyceride splitting activity in the incubation mixture. The tissue factor which brings about this heparin dependent production of clearing factor appears to be most active in lung, heart and pyloric mucosa. Tissue factor has not been solubilized as yet but appears to be localized in the submicroscopic particles of the various tissues studied.

Clearing factor may be assayed by measuring its ability to cause a decrease in the turbidity of alimentary lipemic sera, turbid sera from alloxan diabetic rabbits or synthetic triglyceride emulsions. We have more recently assayed its enzymatic activity by measuring the release of fatty acids and/or glycerol during triglyceride hydrolysis, thus obviating many of the difficulties involved in the measurement of turbidity changes in relatively unstable emulsions (23). In Table 1 are presented the results of an experiment in which fatty acid and glycerol production was followed during the clearing of a cocoanut oil emulsion. The clearing factor employed in this experiment was an alcohol fraction of plasma from which serum albumin had been completely removed by washing the insoluble alcohol precipitate ex-

TABLE 1

CHANGES IN FREE FATTY ACID AND GLYCEROL (HCHO RELEASED BY PERIODATE) CONTENT, AND IN TURBIDITY DURING THE CLEARING OF COCOANUT OIL EMULSION.

Time (min.)	Turbidity (E_{500})	% of max. change	Fatty Acid μM	% of max. change	Glycerol μM	% of max. change
0	0.578	—	4.2	—	0	—
15	0.468	23	8.2	29	0.7	17
30	0.306	56	10.6	46	1.1	26
60	0.194	79	14.9	77	1.7	41
120	0.108	97	16.7	90	2.9	69
180	0.094	100	18.2	100	4.2	100

(Each reaction tube contained 2 ml. cf (plasma), 3 ml. 5% bovine albumin, 4 ml. buffer (30), and 1 ml. 0.5% cocoanut oil emulsion. Incubated at 37° C. One tube analyzed at each time indicated.)

haustively with water. Two major observations can be made from this experiment. First, it is clear that, although fatty acid production follows a more or less parallel course with turbidity change, glycerol production lags considerably behind. This finding indicates the intermediary accumulation of di- and monoglycerides in a nonturbid, solubilized form. Further, the data require the postulation of a solubilizing component for these intermediary products.

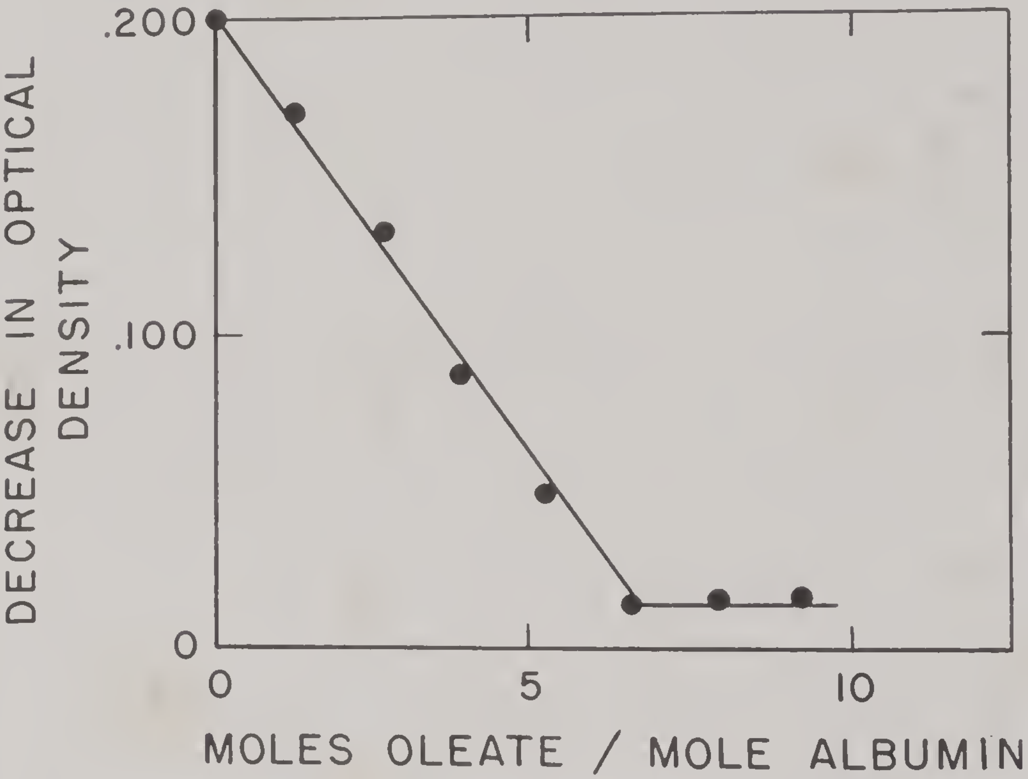

Figure 1. Inhibition of lipemia clearing reaction by oleate.

It will also be noted that the reaction mixture contained added serum albumin, which appears to be an obligatory component of the clearing system. Thus, in these partially purified clearing factor preparations, one can demonstrate that free binding sites on serum albumin, specific for fatty acids, must be available for the normal functioning of the clearing reaction (21). In Figure 1, for example, is shown the results of an experiment in which a purified clearing factor preparation was allowed to attack a cocoanut oil emulsion in the presence of albumin previously treated with increasing quantities of oleate ions. The data indicate that the hydrolysis of triglycerides is essentially completely inhibited when approximately 7 to 8 moles of fatty acid are bound per mole of albumin.

It is possible to demonstrate a further requirement of the system with certain purified preparations of the enzyme. Table 2, for example, shows the marked stimulatory effect of a small amount of normal serum on the catalytic activity of clearing factor in the presence of saturating quantities of serum albumin. The nature of this so-called " coprotein " is, at present, unknown. It is both heat labile and non-dialyzable and therefore presumably protein in nature. The possibility that it is involved in the di- and monoglyceride solubilizing effect mentioned earlier is under investigation.

TABLE 2

EFFECT OF ALBUMIN AND SERUM ON PURIFIED CLEARING FACTOR.

Cofactor supplied to purified clearing factor	Decrease in optical density after 1 hr.	2 hr.
0	.004	.009
.2 cc. 5% albumin	.046	.069
.2 cc. 5% albumin + .005 cc. normal serum	.109	.159

The preliminary chemical and physical observations that have been made permit us to write a generalized equation for clearing factor action as shown in the equation below:

$$(1)\quad \begin{array}{l} \text{Low density} \\ \text{lipoproteins} + \text{albumin} \quad \xrightarrow[\text{(“ coprotein ”)}]{\text{clearing factor}} \\ \text{higher density} \\ \text{lipoproteins} + \text{albumin} \quad (\text{fatty acid})_n + \text{glycerol} \\ \qquad\qquad (+ \text{“ solubilized ” mono- \& diglycerides}) \end{array}$$

Evidence for the normal occurrence of the clearing reaction. Experiments such as those described above indicate that an enzymatic component can be isolated from plasma following heparin injection which has the ability to hydrolyze triglycerides and to mimic the changes which occur normally in plasma after fat ingestion. It has been of particular interest to us to determine whether or not this system plays a normal physiological role in lipid transport.

The requirement for heparin in the production of clearing factor both *in vivo* and *in vitro* has suggested, as one possibility, that heparin may be present as a component prosthetic group of clearing factor. As one means of attacking this problem, we have undertaken the purification of clearing factor to permit direct chemical testing of these preparations for heparin. The experiment in the next table illustrates a method which we feel will yield the purification desired (Table 3). Purification is based on the fact that clearing factor forms a complex with triglyceride emulsions permitting the centripetal separation of the complexed enzyme from other plasma components. As indicated, extensive purification can be obtained in a single step. At present, however, it has not been possible to apply this technique on a large enough scale to obtain sufficient quantities of the enzyme for detailed study.

An inherent difficulty of this method has been its unreproducibility when applied to different plasma samples. This may have been due in part to the competition for clearing factor we have observed between the lipids of low density lipoproteins and of triglyceride emulsions (30).

TABLE 3

PROTOCOL OF A TYPICAL PURIFICATION EXPERIMENT ON CLEARING FACTOR.

8 ml. plasma + 8 ml. 1% cocoanut oil emulsion. Layered under saline in 40 rotor-tube. Centrifuged at 30,000 r.p.m., ½ hr., 5° C. Oil layer resuspended, mixed with starch granules, lyophilized, and oil removed with hexane at –20° C. Enzyme eluted with buffer (equiv. to original plasma volume).

ASSAYS

	$\Delta\epsilon$/30 min/cc	ϵ_{280} (undiluted)	Act/ϵ_{280}	Fold Purif.
Orig. Plasma	0.90	78.6	0.011	—
Eluted CF	0.43	0.36	1.2	108x

TABLE 4

PRELIMINARY STEPS IN THE PURIFICATION OF CLEARING FACTOR FROM DOG PLASMA.

	Volume (ml.)	Units/ml	Mg/ml	Specific Activity	Total * units
Plasma	100	0.63	70	0.9	63
Euglob. fraction	150	0.88	11	8.0	132
5×10^{-4} M. Ba^{++} supernatant	300	0.32	1.1	29.0	96

* The figures in this column suggest the removal of an inhibitory substance during purification.

The complexing procedure is now being applied to preparations on which an effort has been made to remove the variable β lipoprotein components. Table 4 shows the preliminary treatment now employed on active plasma samples before the addition of the complexing emulsion. Step 1 in this procedure is essentially the euglobulin precipitation method of Nikkilä (3). The activity in the supernatant resulting from barium ion treatment is generally 25–30 fold concentrated over plasma and the yield is essentially quantitative.

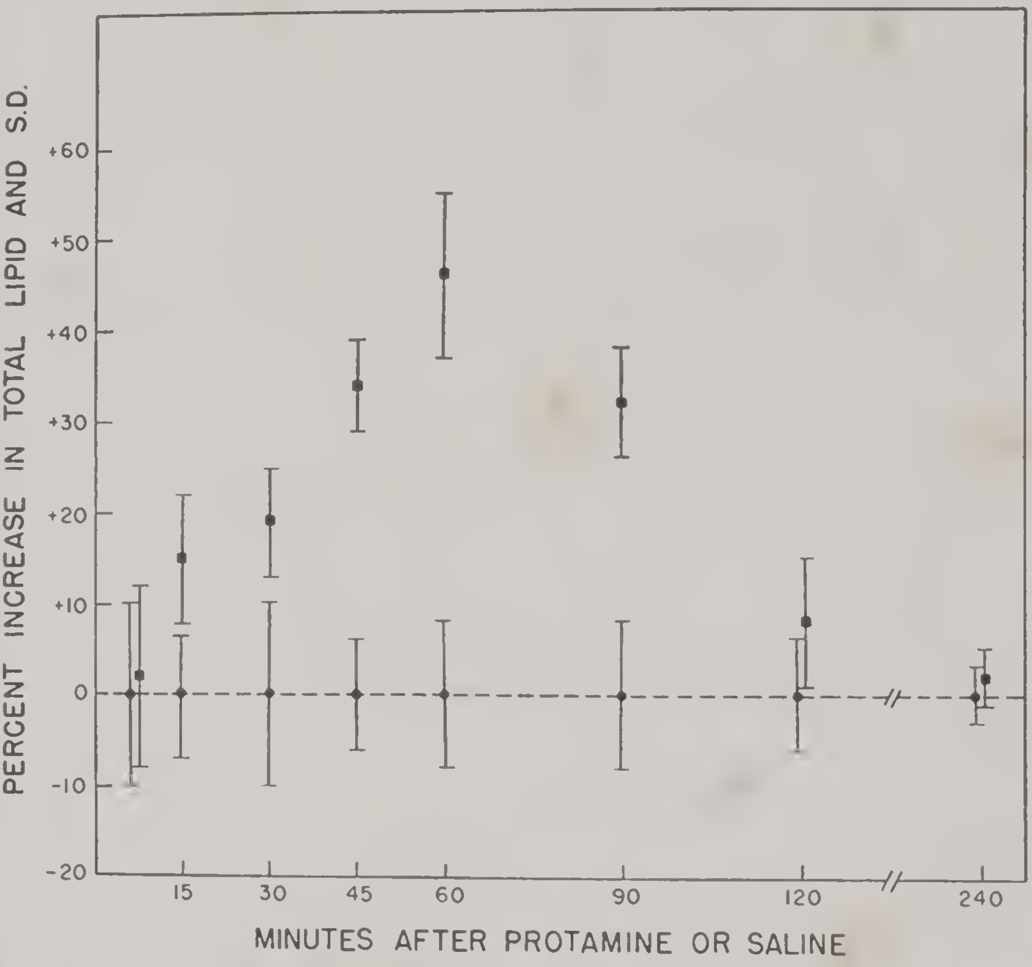

Figure 2. The effect of protamine administration to fasting rats on the level of total plasma lipids.

Various pieces of indirect evidence provide inferential support for the presence of heparin in clearing factor. Several investigators (25–27) have demonstrated that the injection of the anti-heparin agent, protamine, can reverse the clearing effect of heparin in alimentary lipemia. More recently, Drs. Bragdon and Havel (28) have shown that the administration of small amounts of protamine to fasting rats results in a transient increase in the level of plasma lipids with a specific rise in the content of β lipoproteins of the density class S_f 10 and above (Fig. 2). Heparin administration reverses this effect of protamine. These results support the hypothesis that very low levels of clearing factor are present in normal plasma, which are detectable only with difficulty and after considerable concentration (3) by direct methods.

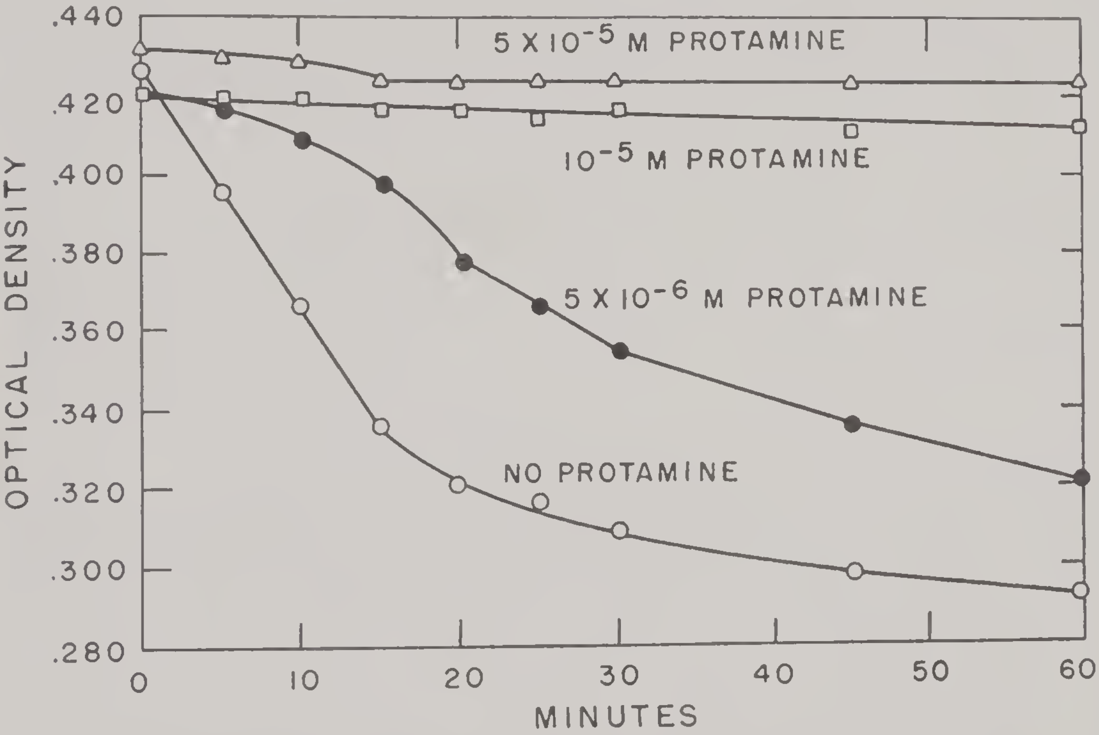

Figure 3. The inhibitory effect of protamine on crude clearing factor activity.

The presence of heparin in clearing factor is also suggested by the inhibitory effect of protamine on crude or partially purified clearing factor preparations. The data in Figure 3 indicate that protamine at low levels can completely inhibit enzymatic activity as measured, in this instance, by the turbidity method on chylomicrons prepared from the serum of alloxan-diabetic rabbits. This inhibitory effect is also shown by the determination of glycerol production as in Table 5. The data in this table also indicate the complete dissimilarity of clearing factor and lipase, the latter being completely unaffected by either protamine or high ionic strength, both of which have marked inhibitory action on clearing factor.

TABLE 5

EFFECT OF HIGH IONIC STRENGTH AND PROTAMINE ON CLEARING FACTOR AND LIPASE ACTIVITY.

Tube No.		μM Glycerol produced/30 min
1	cf control	0.187
2	+ 10^{-5} M protamine	0.090
3	+ 1 M NaCl	0
4	lipase control	0.397
5	+ 10^{-5} M protamine	0.384
6	+ 1 M NaCl	0.417

Clearing factor (cf—15 mg. alcohol fraction (30) per ml. 0.125 M NH_3–NH_4Cl buffer, pH 8.5 preincubated with salt and protamine at levels indicated. 0.4 cc. of preincubated mixture assayed against cocoanut oil emulsion (30). Same incubation procedure used for lipase (steapsin).

Clearing factor is produced in high levels following the administration of anaphylactogenic agents into dogs. These agents presumably cause the release of both heparin and histamine from mast cells. Drs. Havel and Boyle (29) have recently shown that when the shock condition is prevented by various antihistaminic agents, the plasma of these animals contains high levels of clearing factor activity, which disappears upon the subsequent injection of small amounts of protamine. The results of one such experiment are shown in Figure 4.

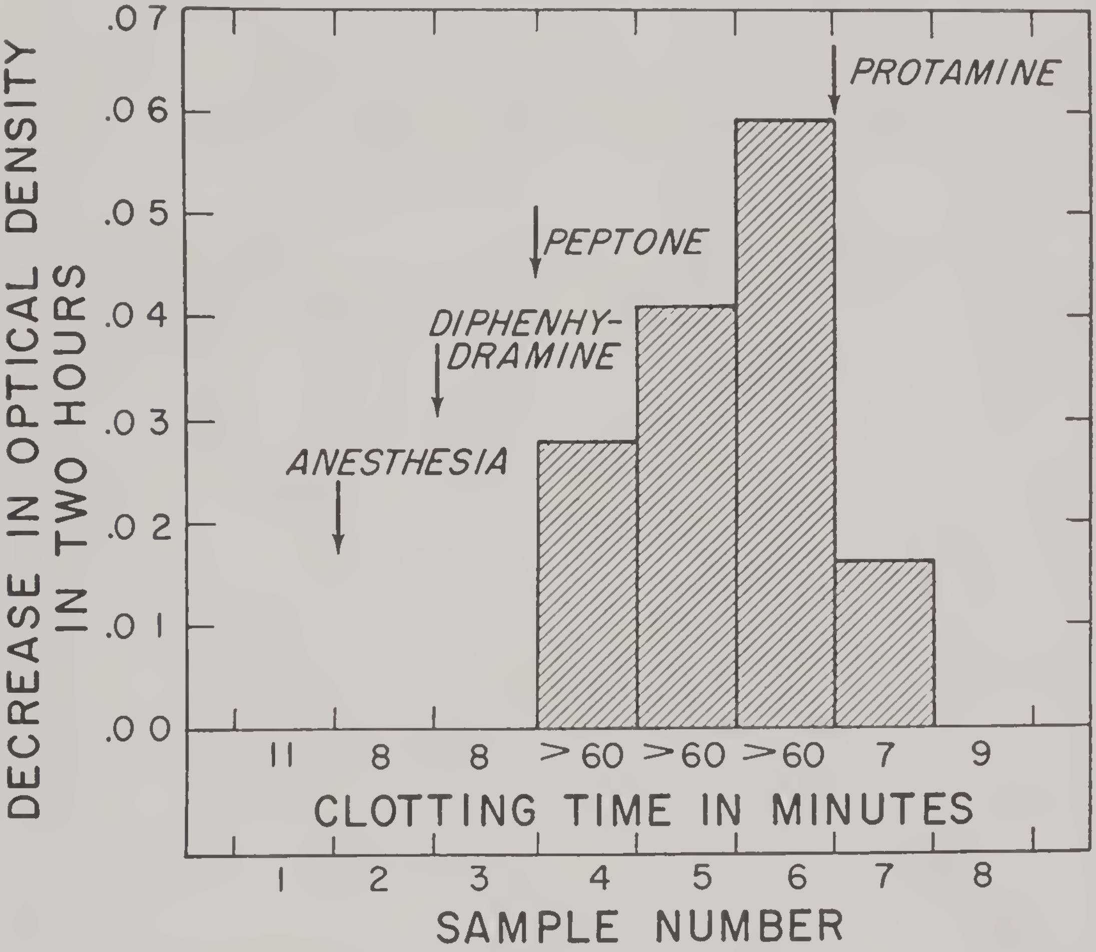

Figure 4. The production of clearing factor activity in the plasma of a dog following the production of shock with peptone. Anesthesia, anti-histaminic, and protamine were injected at the points indicated by the arrows. Plasma samples 1, 2, 3, and 8 contain no clearing factor activity.

In general, therefore, although the presently available data is mainly indirect, it appears not unlikely that mechanisms for the normal production and utilization of clearing factor are present in normal animals.

Products of the clearing factor reaction. Evidence for the production of fatty acids and glycerol and for the probable intermediary accumulation of mono- and diglycerides during the action of clearing factor on low-density lipoproteins has been presented in the above sections. Although it is impossible, at present, to describe the exact fate of the protein moieties of these β lipoproteins, several well-defined physical changes have been studied ultracentrifugally and electrophoretically.

During the action of the clearing factor system on the low-density β lipoproteins of plasma (S_f 10 and above) these components appear to be degraded in the direction of β lipoproteins of higher density (15). Concomitantly, one observes the appearance of high density components with the ultracentrifugal properties of α_1 lipoproteins (17, 18). Such observations suggested originally (30) that the action of clearing factor might be due to " delipidation " with the production of an " α lipoprotein core." Alternatively, the process might involve the transfer of lipid molecules to protein components of plasma with densities originally too great to permit their visualization at the density level of the medium employed for ultracentrifugal analysis. Certain recent data obtained by Dr. Edwin Boyle have shown that the simple addition of long-chain fatty acids can cause the appearance in ultracentrifugal patterns of normal plasma of components with the centrifugal characteristics of both α and β lipoproteins. Although it is clear that these fatty acid-induced components are not identical with the normal α and β lipoproteins present in plasma which contain relatively well-defined levels of phospholipid, cholesterol, and protein, these results do indicate that the interpretation of the ultracentrifugal patterns of plasma should be made with some caution, particularly under circumstances in which fatty acids are being released, as occurs during the course of the clearing reaction. The physical changes induced by the *in vitro* addition of fatty acid to plasma or by the release of fatty acid following heparin injection, have been more thoroughly studied by electrophoretic methods (Dr. R. Gordon—unpublished data). Upon the administration of heparin to individuals whose plasma contains triglyceride-rich β lipoproteins, there occurs a rapid shift in the mobility of a portion of the β globulin peak. Such observations have also been made previously by other investigators, including Nikkilä (3), Rosenberg (31), and Lever et al. (32). In subsequent plasma samples withdrawn from the patient, this shift becomes more marked and the accelerated components may migrate with a mobility even greater than that of serum albumin as shown in Figure 5. With time, this shift is reversed and the electrophoretic pattern assumes its original appearance. Chemical analysis of pre-albumin components removed from the electrophoresis cell indicate a ratio of nitrogen to phosphorus of about 6 : 1, suggesting that the component which is binding the fatty acids released during the clearing are of the β_1 lipoprotein class. The reversion of the pattern to normal suggests the metabolic removal of fatty acid by tissues and raises the possibility that one or more components of the β globulin zone may act as transient carriers of fatty acid. *In vitro* support for this hypothesis is obtained from experiments in which pure S_f 3-8 β lipoprotein was titrated with sodium oleate, producing a marked shift in electrophoretic mobility. This shift was completely reversed by the addition of an approximately equal quantity

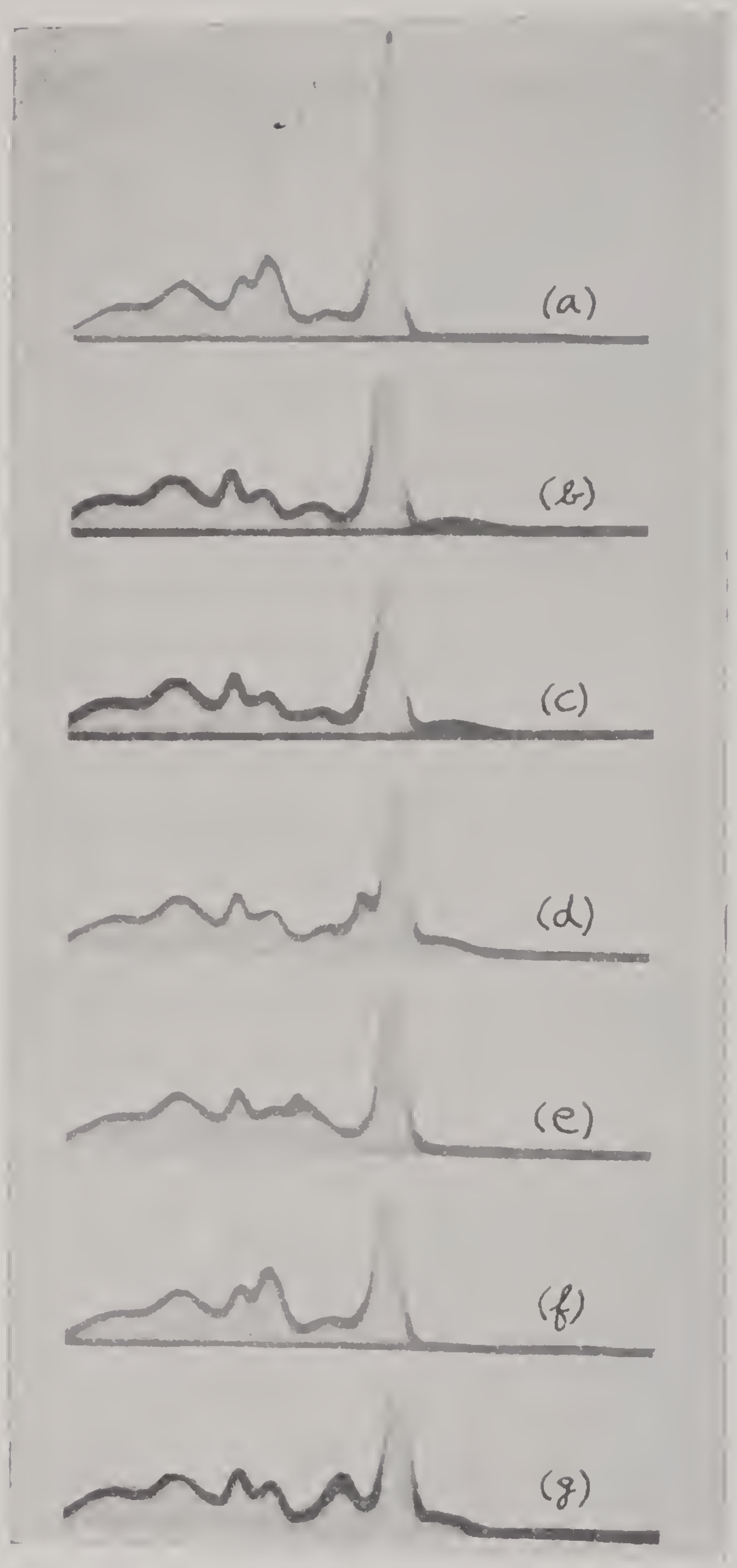

Figure 5. Electrophoretic patterns obtained on plasmas from a hypercholesterolemic patient before (a) and following (b, c, d, e, f) the administration of heparin, 0.75 mg kilo body weight. Pattern (g) is the pattern on plasma sample (a) following oleate addition *in vitro*. Phosphate buffer, pH 7.8, Γ/2 = 0.16.

of serum albumin, which competes more strongly for fatty acid.

In certain hyperlipemic patients with greater initial levels of low density lipoproteins, quite large shifts of mobility may be obtained by incubation, *in vitro*, of plasma samples withdrawn after heparin administration. Under these circumstances, the absence of metabolizing tissues accentuates the shift normally observed *in vivo*. These shifts in mobility occur very rapidly after heparin and the present results indicate the necessity for rapid chilling or salting of the plasma after withdrawal to permit the visualization of early changes. Heparin, in the amounts administered (.75 mg/kg) does not cause a visible change in the electrophoretic mobility of plasma protein components.

General Conclusions

Available evidence suggests that the accumulation of abnormal low density lipoproteins in plasma is the result of deficiencies in the mechanisms for normal degradation of these substances.

Our studies have indicated, in general, that the administration of heparin to individuals, both normal and metabolically disturbed, results in the production of essentially the same levels of clearing factor activity in their plasmas. These findings lead us to feel that, given adequate heparin, the mechanisms for producing and utilizing clearing factor may be generally unimpaired.

By analogy with the hormonal control of the blood sugar level through a homeostatic mechanism dependent on glucose concentration, one is led to consider the possibility that a similar homeostatic mechanism may apply in the case of lipids. The various investigations of the role of heparin in the metabolism of lipoproteins of plasma suggest that a major factor in such homeostasis might be the control of heparin secretion from mast cells, or its biosynthesis. It is felt that a thorough study of this biosynthetic process might lead to valuable advances in our understanding of the regulation of fat transport and mobilization in much the way that our knowledge of blood sugar level regulation has been enhanced by the study of glycogen synthesis and degradation under the action of hormonal agents.

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7. Consideration of the Role of Coenzyme A in Some Phases of Fat Metabolism

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COENZYME A WAS identified as the carrier of reactive acetyl groups (1) just at the time when the field of fat metabolism was ready to be harvested. From isotope experiments it had become most likely that once the nature of the so-called "active acetate" was understood, a very rapid clarification in this whole area would follow. This has happened during the last two years and many laboratories (2) have participated in this mopping-up operation, in particular, after Lynen's (3) work had furnished us with the final detail of the chemical mechanism of acetyl activation.

As an introduction to this section, I would like to show a survey map (Figure 1) from a recent paper by Novelli (4) which illustrates the great diversity of processes in which *CoA* functions. Most of the areas which you recognize on the map will be discussed in greater detail by the speakers and I need not further expand on it. I will rather take this opportunity to make a few special comments about the situation in the area in the center, where fatty acid synthesis is pictured. Using a higher magnification of this spot, we obtain the more detailed picture presented in Figure

2, which also includes an as yet tentative scheme for the early stages in terpene and steroid synthesis.

The initial step there is the condensation of two "active acetates" to acetoacetate. The dependence of this condensation on *CoA* was one of the early observations made in our laboratory (5) which served as an opener to the understanding of fatty acid synthesis. With this initial step we were right away particularly interested in the question of whether we were dealing here with a double activation or if the activation of one acetyl was sufficient. A calculation of the free energy for hydrolysis of acetoacetate to two acetic acids had given a figure of about 16,000 calories (6). This corresponded to the average energy of an energy-rich bond and suggested a single activation to be sufficient, at

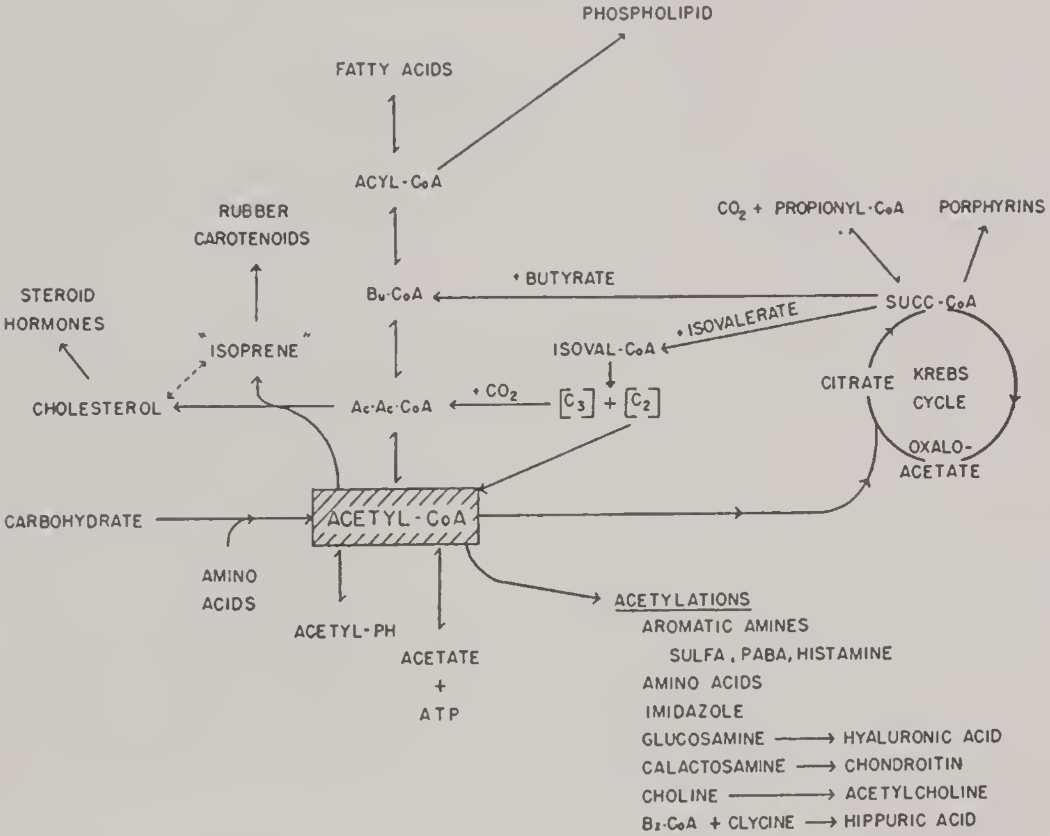

Figure 1. Metabolic Reactions of Acetyl *CoA*.

least energetically. However, subsequently it appeared, somewhat to our surprise, that both " acetates " have to be active to condense (7). It is well known now, mainly through the work of Lynen, Ochoa, Green, and their groups (2) that from the energy point of view a single activation

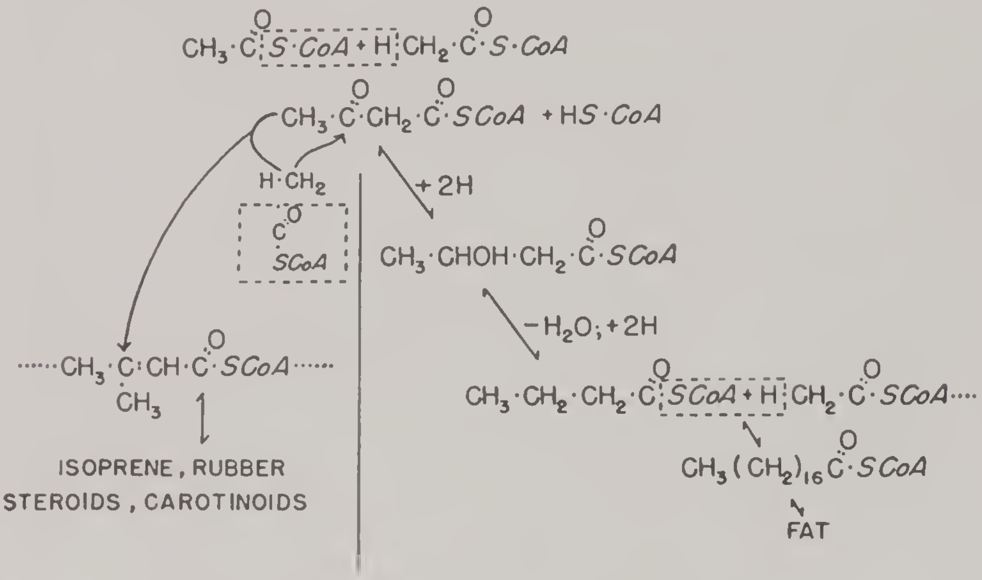
CH₃·C·S·CoA + H·CH₂·C·S·CoA
CH₃·C·CH₂·C·SCoA + HS·CoA
H·CH₂
C
SCoA
+ 2H
CH₃·CHOH·CH₂·C·SCoA
−H₂O; + 2H
······CH₃·C:CH·C·SCoA······
CH₃
ISOPRENE, RUBBER
STEROIDS, CAROTINOIDS
CH₃·CH₂·CH₂·C·SCoA + H·CH₂·C·SCoA····
CH₃(CH₂)₁₆C·SCoA
FAT

Figure 2. Reaction Sequence in Fatty Acid Synthesis.

In the left side of the figure a tentative scheme for steroid synthesis is given. Such appears to involve: 1) A methyl or tail condensation of acetyl *CoA* on the carbonyl of acetoacetate (or *CoA* derivative) to yield β-methyl, b-hydroxy-glutaric acid (dicrotalic acid) (10) and 2) its decarboxylation and dehydration to yield methyl crotonyl *CoA* (11) which, like acetyl *CoA*, may condense head to tail to form longer chains which through reduction and dehydration may eventually lead to formation of polyterpenes.

is sufficient and that the energy of the " second," the terminal carboxyl-*CoA* link is not used in the condensation proper. The reaction product, acetoacetyl *CoA*, retains the energy-rich thioacetyl bond of the second acetyl *CoA* still fully intact. Only the bond energy of the " acceptor acetyl *CoA* " is used and one HS·*CoA* is liberated.

It appeared further that the *CoA* sticks to the carboxyl throughout the whole series of transformations involved in the reduction of the carbonyl to $\cdot CH_2 \cdot$ (*cf.* Figure 2). This whole series of reactions is carried out by enzymes specifically responding to *CoA* derivatives only (8). After thus lengthening the fatty acid chain by two carbons, the *CoA* derivative of the elongated acid emerges and immediately is ready to condense with a new acetyl *CoA* and form the corresponding β-keto acyl *CoA*. This undergoes analogous reduction, dehydration and reduction of the *CoA* derivatives with further lengthening of the chain; and this goes on until the full chain length is obtained. The resulting stearyl or palmityl *CoA*, as Kornberg will explain to you in detail, condenses finally with phosphoglycerol to form the lipids.

The point I want to make is that gearing of this whole reaction phase to *CoA*-linked compounds has an operational or technical rather than a chemical significance,[1] namely to keep the compound ready for subsequent condensation. This, I feel, is an important implication. We often tend to obscure a biological meaning by emphasis on chemical mechanism rather than on process design.

Indeed, just this exclusion of *CoA*-free intermediaries such as plain acetoacetate and hydroxybutyrate from the enzymatic mechanisms of fatty acid synthesis and oxidation appears now to open new insight into the still unsolved problem of ketosis. It is not my intention to present here a theory of diabetic coma. I only want to point out that we are able now to rationalize better the processes leading to a piling up of free acetoacetic acid. This appears largely due to the particular ability of the liver to hydrolyze acetoacetyl *CoA* specifically (9).

The feature, then, of fatty acid metabolism to deal exclusively with *CoA* derivatives makes it appear that the hydrolysis of acetoacetyl *CoA* by liver deacylase " protects " this intermediary from further metabolism and makes it available through the circulation for use throughout the body. How much acetoacetate gets out of the liver will depend, as shown schematically in Figure 3, on the effective

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[1] Most convincing evidence for this is the ease of enzymatic handling of *free* hydroxybutyrate or acetoacetate by the long-known DPN-linked hydroxybutyrate dehydrogenase.

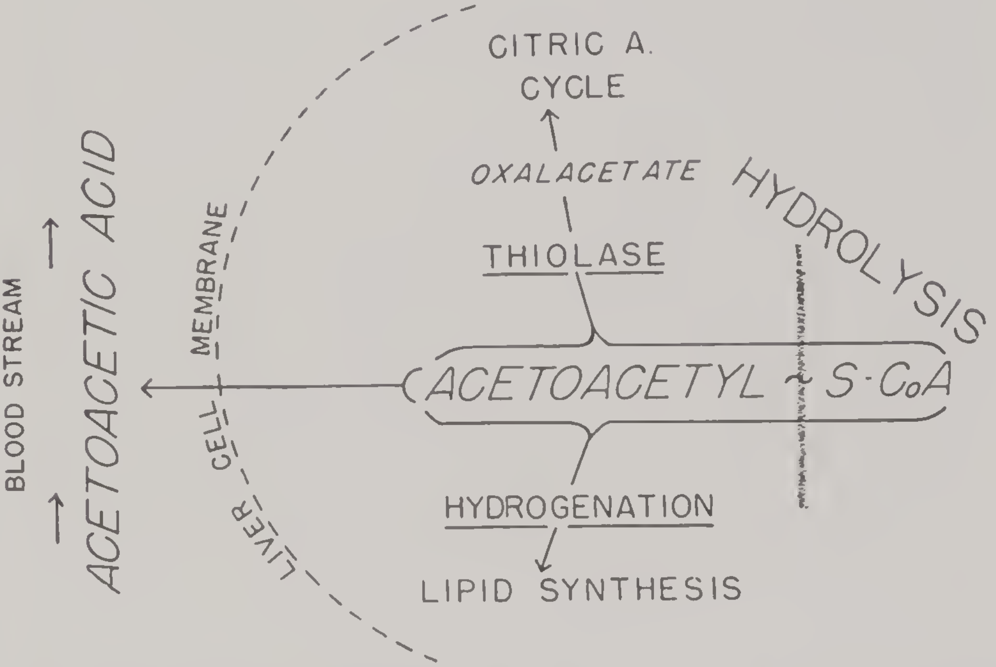

Figure 3. Competition for acetoacetyl·*CoA* by anabolic, catabolic and hydrolytic enzyme systems in liver to illustrate the competitive factors contributing to acidosis.

competition for acetoacetyl *CoA* between oxidation and synthesis on one side, and hydrolysis to free acid on the other. Apparently this balance is relatively easily displaced in the direction of hydrolysis through a weakening of the reductive power of the liver which appears more and more to be one of the major causes for ketosis.

With this discussion, I wanted to emphasize that we shall not expect that every chemical mechanism in the organism serves purely chemical purposes. Indeed, I feel the more we learn to deal in chemical terms with the organism as a whole, the more this just discussed type of " operational design " will come into the picture with quite an important contribution.

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8. Enzymatic Oxidation and Synthesis of Fatty Acids

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THE PRECEDING DISCUSSIONS in this Symposium have outlined some biochemical, physiological, and clinical problems involved in a series of disease states in which aberrations in the metabolism of lipids occur. Before these conditions can be fully and exactly understood in molecular terms it is obvious that we must have information on the nature of the individual reaction mechanisms involved in the intermediary metabolism of lipids and the enzymes catalyzing these reactions. This information by itself will not be enough to interpret physiological and pathological aspects of lipid metabolism. Obviously, in the intact cell, specific controlling mechanisms must exist which regulate the rate of the enzymatic reactions of intermediary metabolism. The mechanisms of this homeostatic control, particularly as exerted by the pertinent hormones, therefore represent another very important area of investigation. Happily the last few years have seen some very great advances in these fundamental areas and it may be hoped that this newly acquired knowledge concerning mechanisms and the new experimental techniques which have evolved may soon be applicable in a rational way to the solution of some of the problems facing the clinical investigator in diseases characterized by aberrations in lipid metabolism.

This paper represents an interpretative account of the very recent and revealing progress which has been made in the intermediary metabolism of fatty acids. Although this discussion will be largely on the level of the intermediary reactions and the enzymes catalyzing them, a few more physiological considerations will also be outlined to serve as a framework of reference.

The last two years have seen an extraordinary burst of experimental success in unraveling the enzymatic mechanisms of fatty acid oxidation and synthesis. The picture we have today, which appears to be almost complete in outline, was literally undreamed of only a few years ago. It was almost exactly ten years ago that enzymatic oxidation of fatty acids was for the first time successfully achieved in a cell-free extract of a tissue by Leloir and Munoz (1). To be able to observe a given sequence of intermediary metabolic reactions in a cell-free extract of the tissue in question is the necessary first step toward understanding the mechanism and isolating the enzymes and coenzymes participating in the process. In the case of fatty acid oxidation, this first step came rather late, as these things go. In the field of carbohydrate metabolism, for instance, fermentation of glucose in cell-free yeast extracts was first observed in 1896. It is obvious that a great deal of progress in fatty acid metabolism has been compressed into a relatively short period of time.

The first experiments of Leloir and Munoz referred to above were not entirely reproducible, but concurrent work in another laboratory (2) pieced out the missing information and soon permitted quite reproducible study of fatty acid oxidation by cell-free liver preparations. In these experiments, an insoluble particulate fraction of rat liver cells, when supplemented with adenosine triphosphate (ATP), Mg^{++}, and orthophosphate, was found to catalyze the oxidation of fatty acids to acetoacetate at the expense of molecular oxygen. The reaction was followed manometrically in the Warburg respirometer. Octanoic acid, the most convenient fatty acid substrate, was found to be oxidized quantitatively to two moles of acetoacetate:

$$(1) \qquad C_7H_{15}COOH + 3O_2 \rightarrow 2CH_3COCH_2COOH + 2H_2O$$

Similarly, such preparations yielded one mole of acetoacetate from one of butyrate and four moles of acetoacetate from one of palmitate. The enzyme preparations did not attack acetoacetate which therefore accumulated quantitatively.

The mechanism of formation of acetoacetate was a point of some interest. Specifically, is acetoacetate formed by fragmentation of the long chain fatty acid directly into 4-carbon units, or does some other mechanism account for acetoacetate formation? This question was convincingly settled by ingenious application of the isotope tracer technique by Weinhouse and his colleagues (3), who studied acetoacetate formation in intact liver slices and later by Buchanan, Gurin, and their colleagues at the University of Pennsylvania, using the cell-free enzyme preparations described above. It was found from these studies that acetoacetate formed by the enzymatic oxidation of carboxyl-labeled octanoate contained isotopic carbon in *both* the carbonyl and carboxyl groups in nearly equal proportion. (Figure 1) The conclusion from these studies was clear and decisive: the fatty acid is oxidized in such a way that it is fragmented into 2-carbon units which must then recombine more or less at random to form acetoacetate.

Another important finding was shortly made. The cell-free enzyme preparations, consisting of particles separated from liver homogenates by centrifugation, were found to catalyze complete oxidation of fatty acids to CO_2 and H_2O via the Krebs tricarboxylic acid cycle if they were supplemented with oxalacetate or a precursor of the latter, in addition to the normal supplements of ATP, Mg^{++}, and phosphate. However acetoacetate is not oxidized via the tricarboxylic acid cycle in this way by liver preparations and it therefore could not have been an intermediate in the complete oxidation of fatty acid to CO_2. On these grounds it was deduced that the hypothetical 2-carbon unit involved in the formation of acetoacetate was probably also directly involved in the condensation with oxalacetate to form citrate and thus permitted fatty acid carbon to enter the tricarboxylic acid cycle (2). See Figure 2. The 2-carbon fragment thus has two pathways open in these cell-free liver preparations. It may be noted that although acetoacetate itself is metabolically inert in such liver preparations, it is oxidized via the tricarboxylic acid cycle in particulate preparations from extrahepatic tissues.

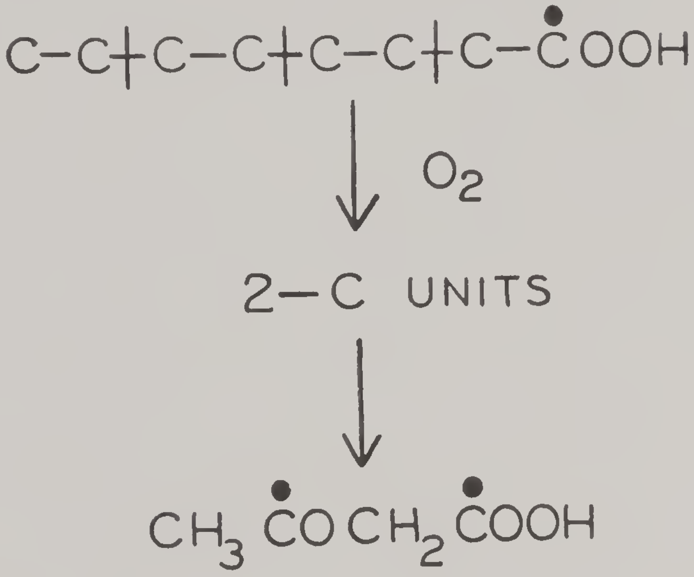

Figure 1. Mechanism of acetoacetate formation.

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It was mentioned earlier that the enzymatic oxidation of fatty acids was catalyzed by a fraction of insoluble particulate elements, separable from liver homogenates by centrifugation. Naturally it was desired to purify this relatively crude system further and to attempt to separate individual enzyme fractions catalyzing only individual reaction steps of the whole cycle. However, such attempts generally led to complete loss of activity. The liver particles were in fact very unstable and lost enzymatic activity even after very mild treatment. It was soon learned that the enzymatic activity of the particles depended on the tonicity of the reaction medium; hypo- or hypertonic reaction media caused

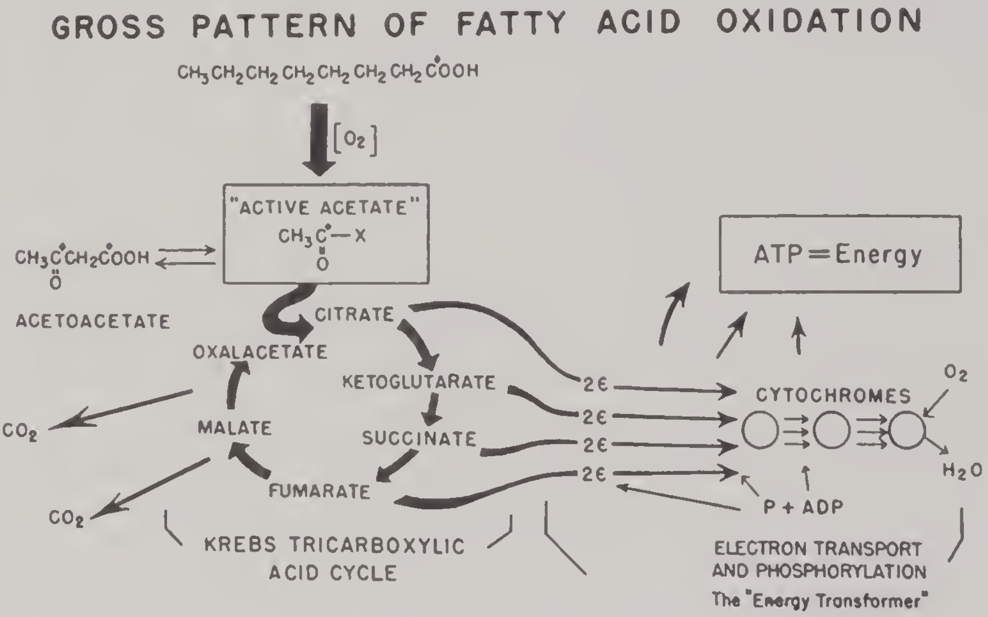


Figure 2 shows not only the gross pattern of the oxidative metabolism of the carbon skeleton of fatty acids but also the fact that during oxidation of the 2-carbon fragment enzymatic dehydrogenations occur and the corresponding electrons are carried over the cytochrome system and ultimately reduce O_2 to H_2O. It is in this process of electron transport that large amounts of energy are released. Through the as yet not well understood mechanisms of oxidative phosphorylation this energy is used to cause the coupled synthesis of ATP from ADP and phosphate.

decreases in the rate of oxidation of fatty acids. These findings very soon led to the discovery in 1948 (*cf.* 4) that the fatty acid oxidase system is contained in the mitochondria of the liver cell, as is the system of enzymes catalyzing the tricarboxylic acid cycle. For some time to come, intact mitochondria of rat liver or kidney, isolated from sucrose homogenates by the now well-known method of Hogeboom, Schneider, and Palade (5), represented the preparation of choice in studying fatty acid oxidation. No success attended efforts to separate the enzymes of fatty acid oxidation in an active form from intact mitochondria until about two years ago. Disruption of mitochondrial structure evidently caused inactivation of proteins or autolytic destruction or dilution of coenzymes.

Further research on the mechanism of fatty acid oxidation as it occurred in intact mitochondria revealed perhaps two more facts of importance. First, it was learned that the carboxyl group of the fatty acid had to be free in order for oxidation to take place, suggesting that the carboxyl group represented a site of enzymatic activation. Second, it was found that neither acetic acid nor any of a list of pertinent 2-carbon compounds would behave as an intermediate in the reactions of enzymatic fatty acid oxidation (2).

Actually, it required some very important developments in a somewhat different though related field of intermediary metabolism to provide the really important key to the picture. This key was the identification of the chemical nature of " active acetate " taking part in certain biological acetylations, in the laboratories of Lipmann and Lynen.

For many years, acetic acid had been suspected to be an intermediate in fatty acid metabolism although direct experimental evidence in support of this view was conspicuously lacking. However, through the work of Bloch and Rittenberg and others of the Columbia school it had been found that isotopic acetate is readily incorporated into fatty acids and cholesterol in intact animals or in surviving tissues, providing conclusive evidence that acetate could be biologically " activated " and then act as an intermediate having the properties of " active acetate." The real key to the problem came out of the work of Dr. Lipmann, our Chairman, who guided a long series of important investigations on the nature of activation of acetate which have affected very radically work and thought in a number of areas of biochemistry. Through this work it was found that acetate is activated in animal tissues by an enzymatic reaction requiring ATP and a new coenzyme, Coenzyme A:

(2) $\text{Acetate} + \text{ATP} + CoA \rightarrow$ " active acetate "

This " active acetate " then could undergo enzymatic reaction with certain acetate " acceptors," such as sulfanilamide and choline, to form acetylsulfanilamide and acetylcholine respectively. Dr. Lipmann and his colleagues ultimately isolated and established the chemical nature of Coenzyme A through a series of arduous and painstaking investigations (6). Its structure is indicated in Figure 3 and it will be noted that implicit in this structural formula is the fundamental biochemical basis for the function of pantothenic acid.

The question now arises: what is the chemical nature of " active acetate "? In 1951 Lynen and his colleagues in Munich reported the isolation of a small amount of " active acetate " from yeast respiring at the expense of added acetate and demonstrated that it consisted of a molecule of acetate in ester linkage with the sulfhydryl group of Coenzyme A (7). "Active acetate " is therefore acetyl-*CoA* (Figure 3) and *CoA* may be regarded as an acetyl carrier, just as adenine nucleotide is a phosphate carrier.

Work in a number of laboratories soon pointed to the unmistakeable fact that acetyl-*CoA* must be the 2-carbon unit involved in fatty acid oxidation. For instance, Ochoa and his colleagues demonstrated that acetyl-*CoA* is involved in citrate formation, a reaction which of course is important in the entry of fatty acid carbon into the tricarboxylic acid cycle:

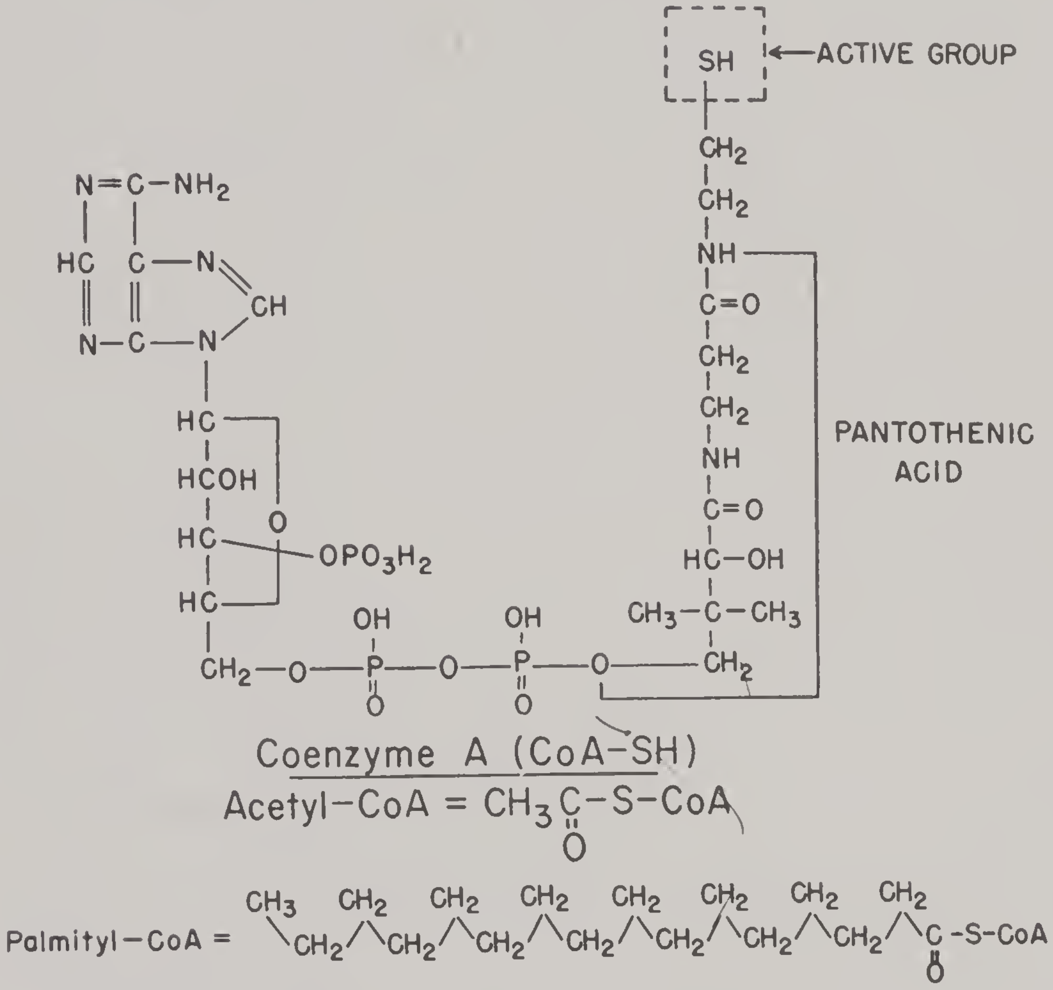
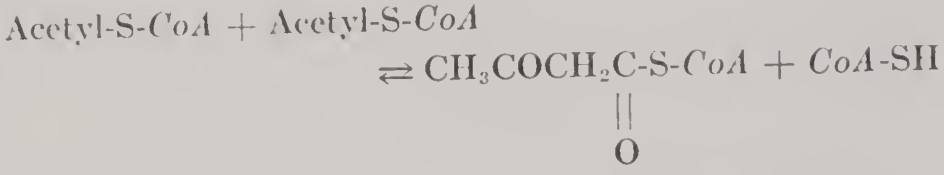


Figure 3. Structure of Coenzyme A.

(3) Acetyl-S-*CoA* + oxalacetate ⇄ citrate + *CoA*-SH

This reaction is catalyzed by the so-called "condensing enzyme," isolated in crystalline form by Ochoa and his colleagues.

The second important reaction is the enzymatic formation of acetoacetate from acetyl-*CoA* which Lipmann, Stadtman, Stern and others have studied (*cf.* 8):

(4) 

$$\text{Acetyl-S-}CoA + \text{Acetyl-S-}CoA \rightleftharpoons \text{CH}_3\text{COCH}_2\overset{}{\underset{\underset{\text{O}}{\|}}{\text{C}}}\text{-S-}CoA + CoA\text{-SH}$$

acetoacetyl-*CoA*

The acetoacetyl-*CoA* thus formed by the reversible combination of two acetyl-*CoA* molecules must then undergo reactions which remove the Coenzyme A with the formation of the free acetoacetate anion. Two such reactions are known: the first is a purely hydrolytic cleavage of the thiol ester linkage by " deacylase " enzymes and the second is a reversible transfer of *CoA* from the carboxyl group of acetoacetate to a carboxyl group of succinate:

(5) Acetoacetyl-*CoA* + $H_2O \rightarrow$ acetoacetate + *CoA*-SH

(6) Acetoacetyl-*CoA* + succinate $\rightleftharpoons$ acetoacetate + succinyl-*CoA*

Succinate is quite specific as *CoA* acceptor in this reversible type of deacylation. We have therefore two mechanisms of deacylation: a purely hydrolytic reaction and a second which has been termed " succinolytic." The full metabolic significance of these two mechanisms of deacylation is not yet understood. In any case it is clearly obvious that acetyl-*CoA* satisfies the requirements of an intermediate in fatty acid oxidation in that it engages in the two most prominent reactions shown by the hypothetical 2-carbon unit formed during the course of fatty acid oxidation.

At this stage it occurred to a number of workers in this field that possibly Coenzyme A plays a role in the oxidation of the fatty acid chain preceding the stage of acetyl-*CoA*; it was suggested that the long-chain fatty acid itself may form a *CoA* ester, analogous to acetyl-*CoA*, and that the fatty acid is enzymatically oxidized in this form (7). Two important experimental facts were consistent with such an hypothesis. First, as was mentioned earlier, the carboxyl group of the fatty acid substrate must be free in order for oxidation to occur. The second observation was that in liver mitochondria preparations and in cell-free extracts of *Clostridium Kluyveri* the process of fatty acid oxidation required an activating stage, sometimes called " priming " or " sparking " (2), under certain experimental conditions. It appeared possible that this activation stage might represent a reaction in which the *CoA* ester of the fatty acid is formed, analogous to the activation of acetate.

The way for investigation of this possibility was opened by the very important finding of Drysdale and Lardy (9) and Mahler (10) that fatty acid oxidation could be observed to occur in clear extracts of mitochondria, provided an artificial electron acceptor system is substituted for the cytochrome system normally functioning in intact mitochondria. Intact mitochondrial structure is therefore unnecessary for the process of fatty acid oxidation under these conditions.

The tempo of research at this point accelerated to a feverish crescendo. Within the past year a shower of " Communications to the Editor " of one or another journal has appeared in which the entire pattern of enzymatic fatty acid oxidation has been outlined in a series of outstanding investigations. These reports are essentially confirmatory of each other and there appears little doubt that they establish the basic enzymatic pattern of fatty acid oxidation. To this development the groups of Lynen in Munich, Green in Madison, and Ochoa in New York have made the major contributions (7), (11), (12).

The results of these efforts are indicated diagrammatically in Figure 4: In the first reaction, the free fatty acid is converted into the *CoA* ester, a reaction similar to that involved in the activation of acetate:

(7) $RCOOH + ATP + CoA \rightleftharpoons RCO\text{-}SCoA + \text{adenylic acid} + \text{pyrophosphate}$

The enzyme catalyzing this reaction has been isolated in highly purified form (14).

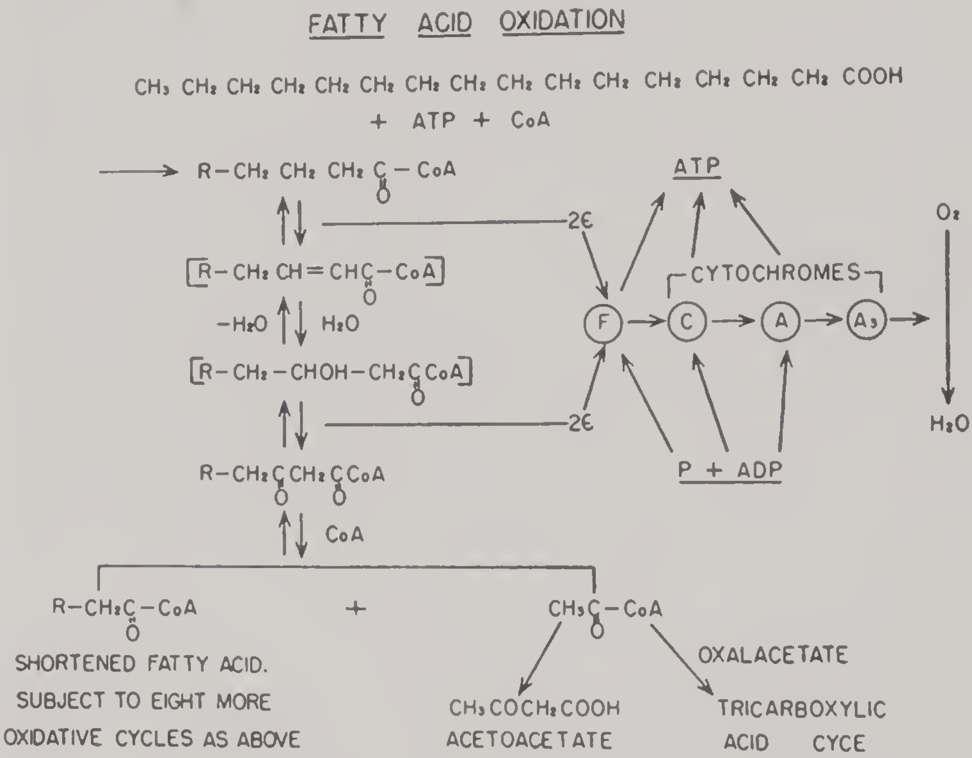


Figure 4. The mechanism of fatty acid oxidation.

In the next reaction, the saturated fatty acid ester of *CoA* is reversibly dehydrogenated at the α, β-carbon atoms to form the α, β-unsaturated acid ester of *CoA*. The dehydrogenase involved has been found to be a flavoprotein containing copper (15). The flavin prosthetic group is reduced in this reaction. Presumably it transfers electrons to the cytochrome system; a reversible Cu^{++} -Cu^{+} step may be the mediator.

In the next reaction reversible hydration of the double bond takes place, yielding the *CoA* ester of a β-hydroxy fatty acid. This intermediate is then oxidized at the expense of DPN^+ by the action of a specific dehydrogenase, again in a reversible manner, yielding a β-keto fatty acid ester of *CoA*, analogous to acetoacetyl-*CoA*.

In the last reaction of the series, a so-called "thiolysis" occurs, in which another molecule of *CoA*-SH, participates and a molecule of acetyl-*CoA* is split off the long-chain fatty acid. This may then enter the tricarboxylic acid cycle or react with another molecule of acetyl-*CoA* to form acetoacetate as already outlined. We are left with a long-chain fatty acid, now shortened by 2-carbon atoms, in the form of its *CoA* ester. This now enters the cycle again. Each time the fatty acid-*CoA* ester is shortened by loss of an acetyl-*CoA* unit and, ultimately, after 7 or 8 such cycles, the 16 and 18-carbon atom fatty acids which predominate in the tissues are completely converted into acetyl-*CoA* molecules. A complete *in vitro* reconstruction of the fatty acid cycle has been achieved in Green's laboratory, in which only the highly purified enzymes and cofactors were employed (12).

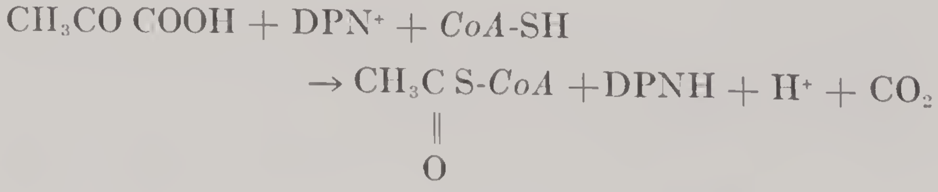
With these advances of the last year or two have come a whole new array of experimental approaches and techniques of measurement, which to the casual reader of biochemical literature probably appear quite formidable in complexity. However, they are generally simple in principle and often employ sensitive spectrophotometric measurements of the characteristic ultraviolet absorption spectra of the *CoA* esters of the certain intermediates (*cf.* 11).

A rather curious detail has come out of this work. It appears from recent work that the β-hydroxy acid ester of *CoA* which is an intermediate in the fatty acid cycle is, quite unexpectedly, the stereoisomer which has generally been regarded heretofore as the "unnatural" form (13). The free β-hydroxybutyrate found in the urine of fasting and diabetic subjects is levo-rotatory; the β-hydroxybutyric acid which is formed as the *CoA* derivative in the fatty acid cycle just described is dextrorotatory. The significance of this unexpected finding is not entirely clear; possibly this situation has a direct bearing on one of the still unsolved problems of fatty acid metabolism, namely the function of free β-hydroxybutyrate in intermediary metabolism. This problem will be referred to again.

It will be noted that all the reactions of fatty acid oxidation as shown are reversible. The equilibria in these reactions are such that the system can readily account for the mechanism of fatty acid synthesis starting from acetyl-*CoA*. In fact, Stansly and Beinert (16) have recently reported that the purified enzymes of the oxidative cycle can catalyze the conversion of isotopically labeled acetyl-*CoA* to butyryl-*CoA* in the presence of the purified enzymes of the cycle and the appropriate reductant system.

Fatty acid synthesis is a process which in its way is just as important biologically as the energy-yielding oxidation of fatty acids. Fat is the only form of foodstuff which may be stored in quantity physiologically and since both carbohydrate and protein are converted to fat, it is obvious that the biosynthesis of fatty acids is a very active process. It now appears clear that acetyl-*CoA* is an obligatory intermediate in the biosynthesis of fatty acids from carbohydrate. Recently Ochoa, Gunsalus, Reed, and others have demonstrated the pattern of the key reaction, the oxidative conversion of pyruvate to acetyl-*CoA*:

(8)



$$\mathrm{CH_3CO\,COOH} + \mathrm{DPN^+} + \mathit{CoA}\text{-SH} \rightarrow \underset{\substack{\| \\ \mathrm{O}}}{\mathrm{CH_3C}}\,\mathrm{S}\text{-}\mathit{CoA} + \mathrm{DPNH} + \mathrm{H^+} + \mathrm{CO_2}$$

The mechanism of this reaction is not yet entirely clear but it is certain that both thiamine pyrophosphate and lipoic acid are necessary components of the pyruvic dehydrogenase system.

It is now appropriate to set this new information on the mechanism of fatty acid oxidation and synthesis into a more physiological framework, both for the purpose of integrating present information on a basis more meaningful to the clinical investigator and also for the purpose of pointing out some still unsolved problems in the intermediary metabolism of fatty acids.

In the first place, all tissues of the mammalian organism which have been examined, and this list includes liver, kidney, heart muscle, skeletal muscle, brain, pancreas, testis, and lung, and some malignant tissues, have the ability to oxidize fatty acids completely to carbon dioxide and water and apparently do so at appreciable rates. It was not so long ago that it was thought that only the liver was of quantitative importance in oxidation of fat. However, only the liver produces significantly high yields of ketone bodies as incomplete products of oxidation of fatty acids. The liver has only a very limited ability to oxidize ketone bodies to CO_2 and H_2O, whereas the extrahepatic tissues readily oxidize the ketone bodies, possibly because they can activate acetoacetate with the formation of acetoacetyl-*CoA* and then acetyl-*CoA*, which of course can " plug into " the tricarboxylic acid cycle. In fasting or diabetes, however, the capacity of the peripheral tissues to utilize ketone bodies is exceeded by the rate of production by the liver, and ketosis intervenes. The ketone bodies may therefore be looked upon as a " trapped " form of acetate which may be transported to peripheral tissues from the liver. However, the recent advances in the enzymology of fatty acid oxidation do not yet give us a satisfactory picture of the physiological control of the formation and utilization of the ketone bodies. The enzymes responsible for deacylating acetoacetyl-*CoA* in the liver and for reactivating free acetoacetate in the extrahepatic tissues must be under some important physiological control. It is conceivable that in this area of ketone body formation and utilization free l-β-hydroxybutyrate may play a special role. It is readily formed from acetoacetate by the action of a well-known and widely distributed dehydrogenase in a reversible manner:

$$\text{(9)} \quad \text{l-}\beta\text{-hydroxybutyrate} + \text{DPN}^+ \rightleftarrows \text{acetoacetate} + \text{DPNH} + \text{H}^+$$

Furthermore, it makes up the bulk of the circulating ketone bodies. It is curious that enzymatic mechanisms exist for the conversion of the l-isomer into the d-isomer (13). Despite this information, no specific biochemical function for the l-β-hydroxybutyrate seems apparent in the light of recent advances. It is conceivable, however, that it represents a transport form of acetoacetate which is less toxic than the latter; keto acids in high concentrations have often been considered to be toxic, perhaps because of a potential ability to combine with -SH groups.

It is hoped that this paper will have served to outline and interpret the recent advances in this very rapidly expanding area of biochemistry.

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DISCUSSION

8a. A Contribution to the Mechanism of Diabetes Mellitus

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AN ENZYMATIC MECHANISM of action of insulin suggests itself from the work of Cori's group (1). They demonstrated that anterior pituitary extract inhibited hexokinase, and that this inhibition could be removed by insulin. This would explain the inability of the diabetic to utilize glucose and the tendency of the diabetic hypophysectomized animal (Houssay dog) to maintain relatively normal glucose metabolism.

Dr. Lehninger then formulated the theory (2) that the cause of the incomplete fat oxidation of the diabetic was a hepatic deficiency in supply of oxalacetate for condensation with the two carbon fragments (acetyl *CoA*) formed from the initial steps of fat oxidation. This deficiency was due to the active oxalacetic decarboxylase of liver. Normally the oxalacetate lost by decarboxylation is replaced by the condensation of pyruvate, derived from glycolysis, with CO_2. Since glycolysis is deficient in the diabetic, due to impairment of the hexokinase reaction, the supply of pyruvate is low and there is no counterbalance to the oxalacetate lost by decarboxylation.

Dr. Lehninger enlarged upon this theory by indicating the requirement of ATP for *activation* of fatty acid oxidation, and phosphate bond energy is derived mainly from the oxidative phosphorylations of the Krebs cycle. This diminution of ATP in diabetic animal tissues was shown by Kaplan and Greenberg (3).

If this were the explanation of the mechanism of diabetes, the administration of certain intermediates to diabetics in failure should relieve ketosis and reverse the entire picture without insulin. Any substitute which could provide pyruvate, or any intermediate of the Krebs cycle, should be effective, provided, of course, that the substrate was utilized.

In the animal or patient rendered ketotic by phlorizin, starvation, fat feeding, or ketone acid administration, any of the above types of substrate is effective in relieving the ketosis. This was studied by many groups in the past and carefully reinvestigated by Beatty et al. (4). The conclusion which can be reached is that any substance which is normally convertible to glucose or to a member of the Krebs cycle can relieve ketosis of the non-diabetic type.

Clinical experience with true diabetics using a number of substrates effective under the above conditions, namely dihydroxyacetone, pyruvate, lactate, citrate, and fructose (5) has shown the inefficacy of the intermediates for relief of ketosis *without insulin.* Our own experience with parenteral succinate, which must have been incorporated into the oxidative cycle since we were able to demonstrate its conversion to glucose, indicates that ketosis in the diabetic is not amenable to the same type of treatment as "carbohydrate starvation" ketosis.[1]

The true diabetic seems to have at least three lesions: one relating to glycolysis, the second impaired ketone oxidation, and the third, supply of ATP for condensation and synthesis. The lesion relating to fat synthesis depends, as shown by Gurin in the following paper, both on ATP supply and glycolysis, and is apparently not specific for insulin, if these requisites are met.

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[1] Beatty and West have subsequently reported data from alloxan diabetic rats which confirm and amplify our clinical experience with succinate (8).

The entire complex of lesions can be explained on the basis of the impairment of the hexokinase reaction if another aspect of this reaction is considered. Lipmann and Lardy demonstrated the need for an *acceptor of high energy phosphate* for maximum efficiency of the oxidative reactions of the Krebs cycle. The hexokinase reaction and the creatine kinase reaction have proven to be the most effective phosphate acceptor reactions in this category, and the hexokinase reaction is used to develop maximum rates of oxidation and phosphorylation by mitochondria.

If we utilize this *second function* of the hexokinase reaction (glucokinase), as shown in Figure 1, as the regulator or stimulator of the Krebs cycle, we can see why no intermediate, including fructose, will be effective. It is not only the substrate which is required, but also the *organization of phosphorylation and acceptor reactions* which must be reestablished in order for the " diabetic " to be brought to a normal metabolic state. Supplying either glycolysis or Krebs cycle intermediates cannot be effective.

The specificity of glucokinase (hexokinase) as contrasted to the fructokinase reaction is that glucokinase (hexokinase) is the ultimate acceptor mechanism. Thus, in the diabetic, even though fructose is taken up more readily than glucose, through the uninhibited fructokinase reaction, the latter does not function fully as an acceptor mechanism, since fructose is readily converted to glucose and this in turn must depend on the glucokinase in order to participate in the acceptor mechanism. The efficiency of the glucokinase acceptor reaction apparently derives from a cyclic regeneration of acceptor by the rapid hydrolysis of glucose 6 phosphate, Figure 1. In this manner glucose can exert far more than a stoichiometric effect. Therefore, only in the presence of a functioning glucokinase reaction will either glucose or fructose be effective in alleviating ketosis.

The site in the Krebs cycle at which this acceptor reaction might be particularly critical is at the ketoglutarate-succinate step where three phosphate bonds are generated and, in addition, succinyl *CoA* is formed, which, as shown by Stern, can activate acetoacetate.

HEXOKINASE AND FATTY ACID OXIDATION

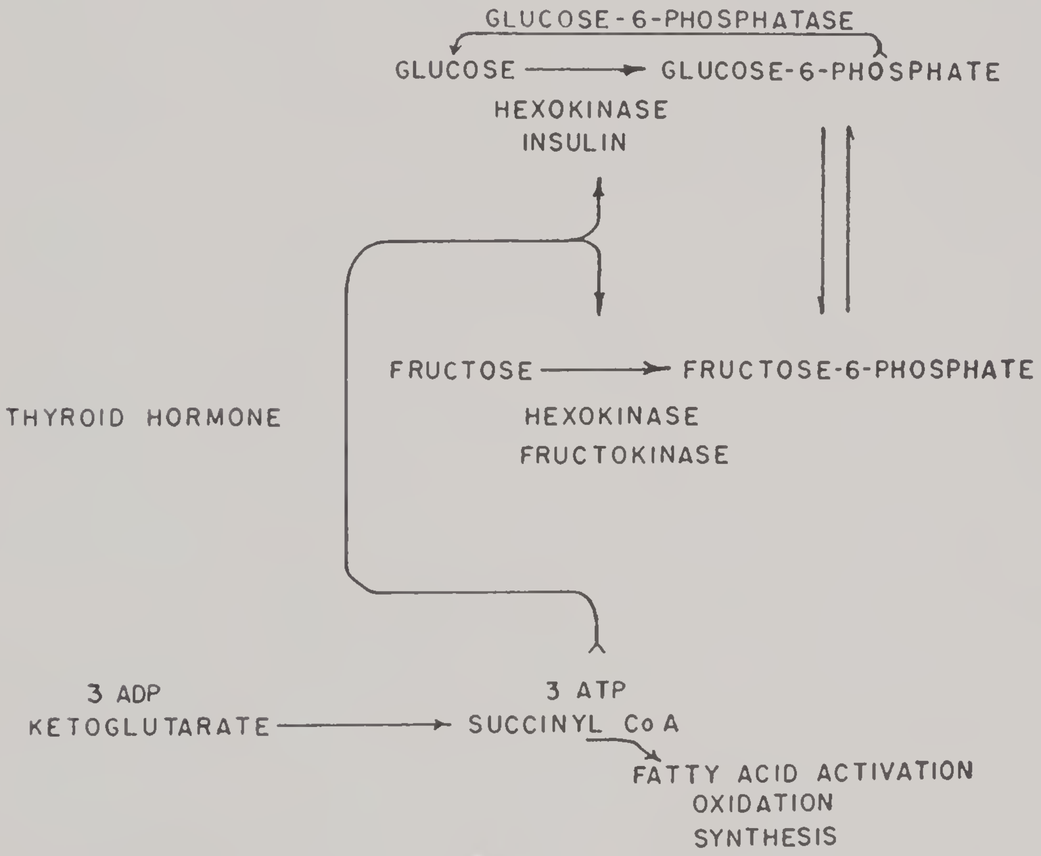

Figure 1

These considerations are shown in Figure 1, which also shows the relation of this effect to the thyroxin effect on phosphate energy transfers demonstrated by Lipmann.

The powerful acceptor system provided by the creatine-ATP reaction in muscle makes muscle independent of insulin for all reactions except glycolysis, and can explain the well-known observation that the oxidation of ketone acids by diabetic muscle is normal.

It must be noted at this point that any indirect effect upon the hexokinase (glucokinase) reaction such as the possibly altered permeability of the diabetic cell to glucose, as suggested by Levine (6) and by Park (7) can explain only the failure of glycolysis.

It is, therefore, proposed that the lesion in the diabetic at the enzyme level is a failure of phosphorylation of glucose through the hexokinase reaction (glucokinase), affecting both glycolysis and oxidative phosphorylation. The hexokinase reaction is thus the most likely site of action of insulin.

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9. Lipogenesis *in vitro* and its Hormonal Control

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THE DEVELOPMENT OF IMPROVED methods for the homogenization of tissues and the separation of cellular components, together with chromatographic and radioisotope techniques have resulted in very rapid advances in our knowledge of lipogenesis. In particular, the separation of the enzymes involved has helped to clarify the metabolic reactions and mechanisms in this complex process.

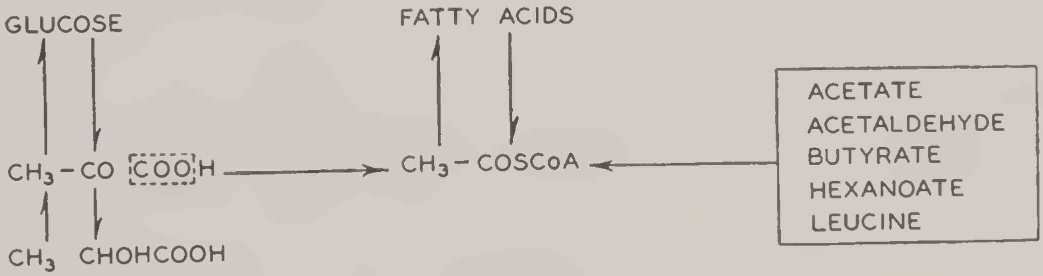

Figure 1.

It is now well established that carbohydrate is converted into fat primarily by way of pyruvate which is subsequently decarboxylated to form acetyl *CoA*. It is reasonably clear that acetyl *CoA* is the major precursor of the long chain fatty acids. The first major metabolic process involved in the conversion of carbohydrate to fat is therefore glycolysis (Figure 1). It follows also that any substance which can give rise to acetyl *CoA* can be converted to fatty acids. A number of investigators utilizing surviving liver slices have, for example, demonstrated that the following labeled compounds can be incorporated into fat: glucose (1), pyruvate (2, 3, 4), lactate (5), acetaldehyde (6), acetone (6), butyric, hexanoic, octanoic (4), and isovaleric acids (6, 7). Similar results with C^{14}-acetate have been obtained by Balmain *et al.* (8) utilizing mammary gland slices, and by Popják and Beeckmans (9) with rabbit intestinal mucosa and lung. Medes *et al.* (10) similarly obtained evidence that rat heart, spleen and testis are capable of performing the same synthetic process. Adipose tissue can also be included in this group (11, 12, 13). It is apparent, therefore, that the ability to synthesize fat is wide-spread. It should be pointed out, however, that all of these positive results simply mean that such tissues contain enzyme systems capable of converting some or all of the carbon atoms of the labeled precursors into fat. Such results do not imply that *net synthesis* has occurred nor do they suggest which of these substances is the important physiological precursor. The amount of fat synthesized by the intact animal for example must be the resultant of a number of physiological factors: diffusion of substrate into the cell, removal of end-products of the reaction, available supply of the right sources of hydrogen and energy. It is attractive to speculate that the action of the hormones may actually lie in this area rather than their acting directly upon individual enzyme systems.

Hormonal influences upon lipogenesis have hitherto been largely studied in the intact animal. The pioneer work of Drury (14) as well as Stetten and his coworkers (15, 16, 17) was done mainly with whole animals. They demonstrated conclusively that while large amounts of carbohydrate are converted into fat by the normal animal, this process is nearly abolished in the "diabetic" state. Both laboratories demonstrated that this inhibition could be reversed by insulin *in vivo.*

The same phenomena can be demonstrated *in vitro* (18). In Table 1 are shown data obtained with surviving liver slices which have been incubated with C^{14}-labeled acetate. If one compares equal weights of liver slices obtained from normal and alloxan-diabetic rats it is seen that the incorporation of labeled acetate into long-chain fatty acids is very materially depressed in the diabetic state. A similar inhibition is observed upon comparison of normal cats with depancreatized cats (18). The addition of insulin to such "diabetic slices" is without effect, nor can any stimulation be obtained by supplementing the slices with glucose, fructose-6-phosphate or α-keto glutarate.

TABLE 1

CONVERSION OF C^{14}-LABELED ACETATE TO FATTY ACIDS BY LIVER SLICES OF ALLOXAN-DIABETIC RATS AND DEPANCREATIZED CATS

No. of Animals	Type of Animal	Micromoles Labeled Acetate Incorporated/100 mg Recovered Fatty Acids
10	Normal rat	10–50
3	Alloxanized rat	0.2–3
6	Normal cat	2–8
3	Pancreatectomized cat (8–60 days postop.)	0–0.7

In general, similar findings have been observed by Chaikoff and his coworkers (5) who employed C^{14}-labeled glucose as the substrate. This laboratory also demonstrated that pre-treatment of alloxanized rats with insulin restored lipogenesis by liver slices (19, 20).

It is interesting to note that insulin does have some effect *in vitro* upon normal liver slices (4). Table 2 demonstrates this effect which has been confirmed by Medes, Thomas and Weinhouse (10) as well as Haugaard and Stadie (21). It should be mentioned that the insulin employed must be reasonably free of hyperglycemic factor which appears to exert an inhibitory effect upon hepatic lipogenesis from acetate (21). Balmain *et al.* (22, 8) have also reported that insulin stimulates lipogenesis when lactating rat and rabbit mammary gland slices are incubated with acetate supplemented with glucose. A similar effect with lactating rat

TABLE 2

CONVERSION OF CARBOXYL OR METHYL LABELED ACETATE TO FATTY ACIDS BY RAT LIVER SLICES AND IN VITRO EFFECT OF INSULIN

Expt. No.	Substrate	Insulin	Micromoles Substrate Incorporated/100 mg. Recovered Fatty Acids
1	$CH_3C^{14}OONa$	—	17
		+	38
2	$CH_3C^{14}OONa$	—	7
		+	13
3	$C^{14}H_3COONa$	—	11
		+	17
4	$C^{14}H_3COONa$	—	11
		+	17
5	$C^{14}H_3COONa$	—	9
		+	14
6	$C^{14}H_3COONa$	—	21
		+	30
7	$C^{14}H_3COONa$	—	23
		+	37

mammary gland slices has been observed by Hills and Stadie (23).

It is not too surprising that the nutritional state of the organism has a profound effect upon lipogenesis. Masoro *et. al.* (20) have observed that fasting for 24 hours was sufficient to reduce the incorporation of glucose into fat (by liver slices) to less than one-tenth of that observed on a standard dietary. We have obtained similar results with labeled acetate as have Lyon *et al.* (24) and Van Bruggen and coworkers (25). It is interesting that even a change in dietary from high carbohydrate to one high in protein or high in protein and fat resulted in some inhibition of lipogenesis (20). Such results indicate quite clearly, that subsequent studies of the influence of hormones upon lipogenesis must take into account the nutritional status of the experimental animal. Before taking leave of this aspect of the problem, it should be noted that inhibited lipogenesis brought about by fasting can be overcome by the addition of fairly large amounts of glucose or glucose plus insulin (20).

Liver slices from hypophysectomized-pancreatectomized cats (Table 3) are able to convert labeled acetate into fat at a normal rate (26). It would appear, therefore, that lipogenesis can proceed in the absence of insulin provided the hypophysis is also removed. The addition of insulin to such slices does have some stimulating effect. If purified growth hormone (free of ACTH) is administered to such Houssay animals for a few days prior to sacrifice, lipogenesis by liver slices is again retarded. Liver slices from adrenalectomized-pancreatectomized animals also appear to retain their ability to synthesize fat. It should be mentioned, however, that impaired lipogenesis was observed in the case of two such animals which appeared to have an unusually high liver fat content.

Prior injection of cortisone to normal rats for 3–4 days produces livers which have lost to a considerable extent their ability to convert acetate to fatty acids (26). Balmain and coworkers (27) have also demonstrated an inhibition of lipogenesis when cortisone is added *in vitro* to rat mammary gland slices. This laboratory also reported *in vitro* evidence suggesting an antagonism between insulin and cortisone. The results obtained by both groups are consistent with the observations of Welt and Wilhelmi (28) that both growth, horomone and ACTH, depress lipogenesis in the intact animal.

TABLE 3

CONVERSION OF C^{14}-LABELED ACETATE TO FATTY ACIDS BY CAT LIVER SLICES

No. of Animals	Condition	Micromoles Labeled Acetate Incorporated/100 mg. Recovered Fatty Acids
7	Houssay (4–43 days, Postop.)	2–9
2	Houssay + growth hormone	0–0.7
5	Adrenalect.-Pancreatect. (3–11 days, Postop.)	0.3–5

The inability of the “diabetic liver” to synthesize fat cannot be ascribed to a lack of acetyl *CoA*. Fatty acid oxidation proceeds at an accelerated rate in the diabetic state as it does in fasting (Figure 2). The work of Stadie's group and others is too well known to bear repetition. Weinhouse and coworkers (29) have similarly shown a high rate of ketogenesis in liver slices of fasted animals incubated with labeled fatty acids. The acetyl *CoA* resulting from this oxidation of fatty acids may be handled in several ways:

(1) condense with oxalacetate to form citrate (30–34) and subsequently be oxidized by way of the Krebs cycle to CO_2 and H_2O; (2) undergo self-condensation to form acetoacetyl *CoA* which is rapidly hydrolyzed by liver yielding acetoacetate; (3) be utilized for the biosynthesis of cholesterol. In the fasting or diabetic state there is presumably then no lack of 2-carbon fragments nor is there a failure of the Krebs aerobic cycle. Energy sources of some kind are therefore available. Why is there a failure of lipogenesis in these two physiological states? Chaikoff and his coworkers believe that efficient lipogenesis by liver requires the simultaneous participation of glycolysis. Baker *et al.* (35) have demonstrated that prior ingestion of fructose by alloxan-diabetic rats restores the ability of the liver slices to form fatty acids from labeled acetate but *not from C^{14}-glucose*. This is quite different, of course, from the effects of administration of insulin which restores lipogenesis from glucose. Chernick *et al.* (36, 37) have demonstrated that diabetic and normal liver slices utilize about the same amount of fructose. Therefore, fructokinase and the remaining glycolytic enzmes appear to be relatively intact in the diabetic state. Since there is

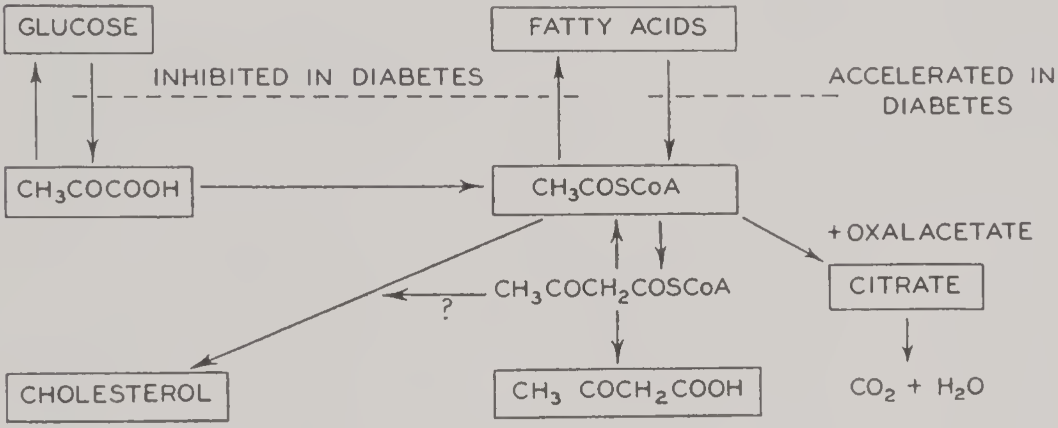

Figure 2.

reasonable evidence that the Krebs cycle is not significantly impaired provided pyruvate, lactate or acetate are the substrates employed, it is clear that both the glycolytic and oxidative enzymes are relatively unimpaired in the diabetic state. The activation of acetate presumably to acetyl *CoA* appears to be normal in the fasting state as well (10).

One can well argue that a common feature of diabetes and the fasting state could involve a deficiency of glycolytic intermediates. If this is so, then the addition of phosphorylated intermediates of glycolysis to a cell-free system prepared from diabetic liver should restore lipogenic activity. This has been shown to be the case (38). It has recently been found possible to prepare aqueous particle-free extracts of liver capable of converting acetate or pyruvate to long-chain fatty acids (39, 40). Incidentally, it was comforting to find a system (Table 4) in which pyruvate served as a better source of fat than did acetate. The cofactor requirements for this enzyme system are Mg^{++}, ATP, DPN, *CoA* and probably a flavoprotein (41). Relatively high concentrations of citrate (0.1–0.2M) are necessary for maximal activity (40).

TABLE 4

INCORPORATION OF 1-C-14 ACETATE AND 2-C-14 PYRUVATE INTO LONG CHAIN FATTY ACIDS BY PARTICLE-FREE EXTRACTS OF RAT LIVER

Substrate	Concentration	*μM*. Incorporated
Acetate	35 *μM*.	0.04
"	35 *μM*.	0.04
Pyruvate	17.5 *μM*.	0.14
"	35 *μM*.	0.23
"	52.5 *μM*.	0.38

In Table 5 are shown the results obtained with aqueous extracts derived from livers of alloxan-diabetic rats. In agreement with the results reported on the whole animal and liver slices, these extracts are no longer capable of synthesizing fatty acids effectively. If mitochondria obtained from normal rat liver homogenates are added to such a system

TABLE 5

LIPOGENESIS BY PARTICLE-FREE EXTRACTS OF ALLOXAN-DIABETIC RAT LIVER

The complete system consisted of 4.5 ml. of enzyme preparation, 0.01 M K citrate (final concentration), 54.5 μmoles of pyruvate-2-C^{14} (1.25×10^5 counts/min/mg C.) plus the additions indicated below. The total volume, 5 ml; gas phase, air; incubation time, 3 hr.; temp. 34° C.

Preparation	*Additions*	Recovered fatty acids			
		Counts/min/mg fatty acid		Micromoles of pyruvate incorporated/10 mg fatty acid	
		1	2	1	2
1. Normal	None	82.0	99.6	0.18	0.21
Diabetic	None	5.6	13.3	0.01	0.03
Diabetic	Normal mitochondria	6.1		0.01	
Diabetic	Normal supernatant	18.9	31.6	0.04	0.07
Diabetic	Glycogen, 50 mg.	32.2	49.8	0.07	0.11
Diabetic	Hexose diphosphate, 11 μmoles	51.5	62.5	0.11	0.13
2. Diabetic	None	8.0	4.4	0.02	0.01
Diabetic	Glucose, 11 μmoles	7.8[a]		0.02	
Diabetic	Glucose 6-phosphate, 11 μmoles	15.6	11.0	0.03	0.02
Diabetic	Glucose 6-phosphate, 11 μmoles + 1 mg. ATP[b]		27.1		0.06
Diabetic	Hexose diphosphate, 11 μmoles		48.0		0.11
3. Diabetic	None	5.7		0.01	
Diabetic	Fructose, 11 μmoles	21.2[a]		0.05	
Diabetic	Hexose diphosphate, 11 μmoles	50.2		0.11	

a. These results have been confirmed several times.

b. Adenosine triphosphate.

there is no effect. In contrast, supernatant fluid from such a homogenate is stimulatory. The addition of glycogen or hexose diphosphate produced even greater effects. Whereas the addition of glucose to such " diabetic liver extracts " was without effect, glucose-6-phosphate, glucose-6-phosphate plus ATP, hexose diphosphate and fructose were strikingly effective. In other words, a solution which was unable to respond to the addition of glucose, was capable of being stimulated by fructose, glycogen or phosphorylated intermediates of glycolysis. These results certainly suggest that we may be dealing here with a block in the phosphorylation of glucose. They appear also to favor the hypothesis that lipogenesis is dependent upon efficient glycolysis.

In a further study of glycolytic intermediates ribose-5-phosphate and phosphopyruvate plus ADP have also been found to be effective. In any event, it is obvious that lipogenesis cannot occur in the diabetic animal unless glucose is effectively metabolized by the Meyerhof-Embden cycle or by some other pathway.

It is clear that the diabetic animal accumulates fat in the liver because the fat is brought there from extra-hepatic sources. It obviously cannot be synthesized there. So far as *in vitro* experiments go, we know that diabetic liver can oxidize fatty acids at an accelerated rate (Figure 2). The acetyl *CoA* which is thus rapidly formed may saturate the available oxalacetate. Excess acetyl *CoA*, since it cannot be re-converted back to fat, has only two places to go: to acetoacetate and to cholesterol.

The metabolic effects of a lack of insulin may therefore be very far-reaching. The failure to utilize glucose involves an attendant lack of glycolytic intermediates and pyruvate. A deficiency of this sort impairs lipogenesis; it may also result in a relative deficiency of oxalacetate. This would explain the ketosis associated with the diabetic state; it could explain the increased rate of cholesterol synthesis. The mechanism by which this and other hormones operate may well be to displace equilibria; that is remove end-products or conversely to prevent substrates from arriving at the scene of action. It may therefore be futile to look for direct *in vitro* effects of hormones upon isolated enzyme systems. It will be of interest in the future to prepare cell-free systems obtained from animals suffering from a deficiency or excess of some particular hormone. With some perseverence it should at least be possible to determine what metabolic abnormality has resulted. Although final answers regarding the exact mode of action of the hormones may not be forthcoming, this type of experimentation should provide some interesting clues.

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10. Lipid and Phospholipid Synthesis[1]

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I HAD WONDERED ABOUT the function of a microbiologist in this symposium which brings together clinicians and biochemists in an effort to improve the understanding of a metabolic disorder in higher animals. Perhaps I should bring you a message based on the precepts of one of the founders of modern microbiology, Robert Koch. In his now famous postulates, he pointed out that in order to establish that a micro-organism is the responsible agent of a disorder, it is necessary (1) to find it in the lesions of the diseased host, (2) to isolate the micro-organism in pure culture, (3) to reproduce the disorder upon inoculating a susceptible host with a sample of the pure culture and, finally, (4) to re-isolate the micro-organism again from the newly-produced lesions. In an analogous way, it appears to me that in trying to establish the nature of a metabolic phenomenon, one must (1) find the enzyme system in the cell or tissue demonstrating the phenomenon, (2) isolate the enzyme system in as pure a state as possible, and then (3) show that the purified enzyme system alone, or integrated with others, can reproduce this phenomenon. Perhaps such a doctrine would indeed unify the efforts of the biochemist and his clinical colleagues.

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[1] For recent comprehensive reviews of this and related subjects see references 1, 2 and 3.

Within the last few years, the efforts from several laboratories taken together have permitted the beautiful detailed reconstruction of the synthesis and degradation of fatty acids, which has just been reviewed by the previous two speakers. This achievement, which far exceeds the most optimistic predictions of earlier biochemists, underlines three points which are pertinent to the problem of lipid and phospholipid metabolism, as well as to many other metabolic problems.

First, the metabolism of the most complex molecules can and should, despite their complexity, become the subject of detailed enzymatic investigation.

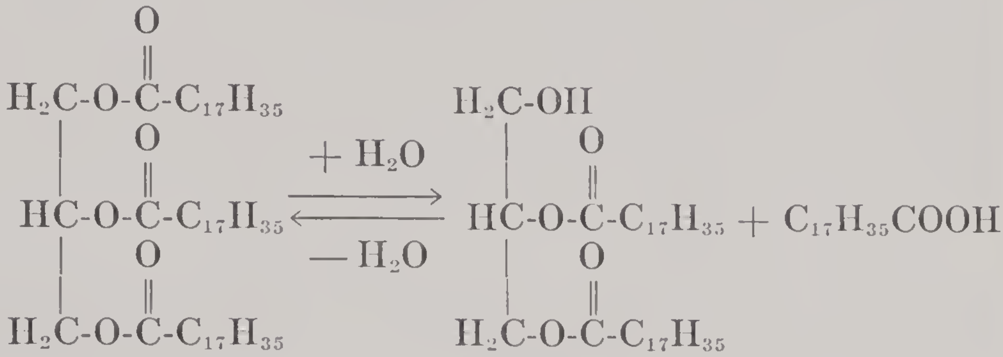
Secondly, large, complicated molecules are pieced together out of relatively small fragments by an orderly, stepwise, enzymatic process.

And, finally, it is profitable to explore the degradative metabolic pathways as likely routes of synthesis. Despite the intense congestion on many metabolic highways, nature has often managed to regulate the traffic without installing one-way streets. In fact, the routes of carbohydrate and fat metabolism, which are now among the best understood in intermediary metabolism, are composed for the most part of readily reversible reactions.

Lipases and Esterases

With this last point in mind, let us briefly review what is known about the enzymatic degradation of lipids and phospholipids and try to determine whether any of these reactions are responsible for synthetic events in the organism. Shown in Equation 1 is a common and typical fat, tristearin, which consists of glycerol esterified at each of its three alcohol groupings with a molecule of fatty acid, in this case the 18-carbon, stearic acid. When acted upon by an ester-splitting enzyme, such as the lipase of pancreas or liver, the H and OH of water are added across the ester bridge to yield alcohol and fatty acid.

Equation 1

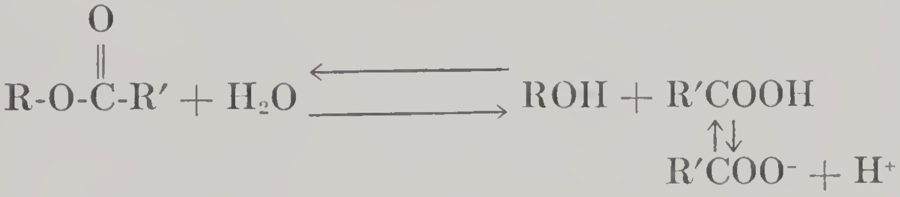


$$\begin{array}{l} H_2C\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_{17}H_{35} \\ \;| \\ HC\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_{17}H_{35} \\ \;| \\ H_2C\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_{17}H_{35} \end{array} \underset{-\,H_2O}{\overset{+\,H_2O}{\rightleftarrows}} \begin{array}{l} H_2C\text{-}OH \\ \;| \\ HC\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_{17}H_{35} \\ \;| \\ H_2C\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_{17}H_{35} \end{array} + C_{17}H_{35}COOH$$

Tristearin Distearin Stearic Acid

Equation 2 shows a more generalized formulation.

Equation 2



$$R\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}R' + H_2O \rightleftarrows \begin{array}{l} ROH + R'COOH \\ \qquad\quad \uparrow\downarrow \\ \qquad\quad R'COO^- + H^+ \end{array}$$

Alcohol + Acid

Were it not for two factors in this equation, the reaction would be freely reversible, as likely to make fat as break it. One factor is water, which, in all receipes of physiological media, is present at a concentration over one thousand times as great as glycerol or fatty acid. The second factor is the H^+ concentration which, at body pH, is less than a thousandth as great as that of the fatty acid and glycerol. In other words, the overwhelming concentration of water on the left side, and the exceedingly low H^+ concentration on the right side, would force the reaction equilibrium very far to the right and render fat synthesis rather unlikely.

Yet there has been a great deal of work with *in vitro* systems which does demonstrate the synthetic action of lipase. An interesting example is the well-known esterification of cholesterol by serum or pancreatic extracts. In a mixture of cholesterol, fatty acid and crude pancreas powder, over half of the cholesterol can be esterified in a few hours (Table 1). Similar observations have been made with serum esterase. Bile salts, which greatly accelerate the reaction, appear to serve a catalytic function in the reaction. A plausible interpretation is that we are dealing here with a nonhomogeneous, two-phase system, the esterification taking place in the cholesterol phase which dissolves undissociated fatty acid but excludes water. It is important to emphasize that the cell is not a homogeneous system either, and within the organized structure of certain cells water-poor areas as well as actual fat droplets exist. It is a question for future work to determine whether lipases and esterases do perform a synthetic function *in vivo* and, if so, to what extent and under what restrictions. Regardless of the solution to this

TABLE 1

ESTERIFICATION OF CHOLESTEROL BY A PANCREATIN PREPARATION (Swell and Treadwell (4))

Experiment	Sodium Taurocholate mgs.	Esterification of Cholesterol 6 hrs.	Esterification of Cholesterol 24 hrs.
		%	%
1	0	0.0	0.0
2	50	35.2	67.2
3	100	54.9	76.5

Incubation mixtures contained 75 mg. of cholesterol, 164.4 mg. of oleic acid, 200 mg. of Merck pancreatin, U.S.P., the stated amount of sodium taurocholate, and 500 mg. of egg albumin emulsified in 13 ml. of phosphate buffer (0.12 M, pH 6.6). Tubes were shaken in a bath at 37°.

question, it does seem rather likely from general considerations that systems comparable to those involved in the synthesis of phospholipids (discussed below) are also operative in neutral fat synthesis.

Phospholipases

With regard to the breakdown of phospholipids, there is in nature a wide variety of enzymatic mechanisms for its breakdown. Let us consider lecithin as an example (Figure 1). Lecithin bears some resemblance to neutral fat in that glycerol is esterified by long-chain fatty acids. However, only two fatty acids are present and generally one, and sometimes both of them, are unsaturated. The third group of glycerol is linked to phosphate which in turn is esterified to choline.

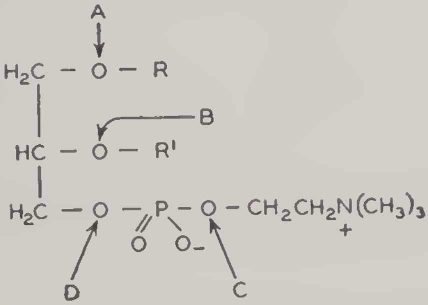
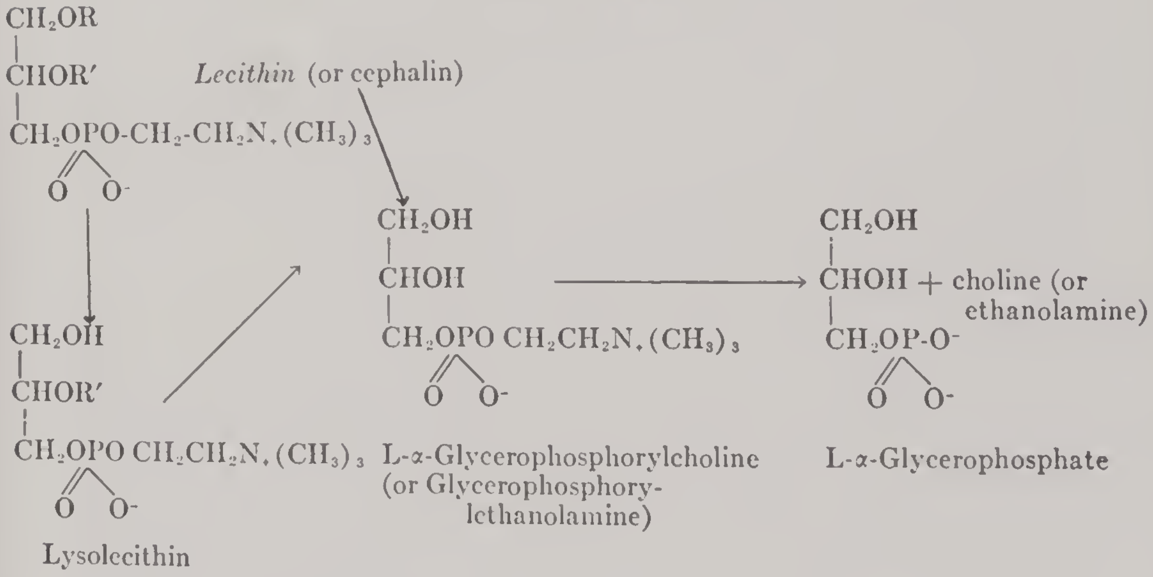


Figure 1. Sites of Cleavage by Various Phospholipases.

Enzymes have been described which attack each of the indicated linkages. Thus far the splitting of lecithin at point C to yield free choline and a phosphatidic acid has been observed only in plants. Initial splitting at D to yield phosphorylcholine and a diglyceride is the well known action of *Clostridium welchii* (perfringens) toxin; it has also been described in snake venom but not in higher animals.

Splitting of lecithin at A or B to yield a free fatty acid and lysolecithin is an enzymatic activity which is widespread in nature. Schmidt *et al.* (5) and Kahane and Lévy (6) have shown that the breakdown of lecithin in autolyzing tissues seems to proceed by the action of phospholipases A and B, and leads to the transient accumulation of glycerophosphorylcholine. About two years ago, Hayaishi and I (7) isolated a bacterium from soil which is particularly proficient in the metabolism of lecithin. With purified enzymes from cell-free extracts of this organism, we determined that the primary attack on lecithin, as indicated in Equation 3, was the release of fatty acids with an accumulation of glycerophosphorylcholine (GPC). Indications are that lysolecithin is the intermediate in this conversion. We isolated a second, very active enzyme from this organism which splits GPC to yield L-α-glycerophosphate and choline. Completely analogous results were obtained with cephalin and the glycerophosphorylethanolamine derived from it. It is pertinent at this point to mention a report by Shapiro (7a) presenting evidence for the accumulation of an acylated intermediate in the course of lecithin splitting by pancreatic preparations.

EQUATION 3



Interesting and varied as these patterns of phospholipid metabolism are, there has as yet been no demonstration that any of these reactions is reversible and could provide a possible route of phospholipid synthesis.

Phospholipid Biosynthesis

For the remainder of this discussion, I would like to consider the biosynthesis of phospholipids. There is, as you know, an abundant literature on the *in vivo* metabolism of phospholipids, a literature that was greatly enriched by the availability of the radioactive isotopes of phosphorus and carbon. The use of P^{32}-labeled inorganic phosphate, glycerophosphate and phosphorylcholine, and of C^{14}-labeled choline, demonstrated that, as in the case of protein, there is no large fraction of body phospholipids which is fixed, structural and metabolically inert. Rather there appears to be a rapid and inevitable turnover of lipids and phospholipids, under the influence of a variety of nutritional and physiologic factors. As for the mechanism whereby phospholipids are assembled, relatively little has been learned from these studies. The work of Fries *et al.* (8), Friedkin and Lehninger (9), and Artom *et al.* (10) with broken cell preparations from liver and brain did show that single units, such as choline and inorganic phosphate, were incorporated into the phospholipids in the absence of intact cell structures.

Our interest in phospholipid synthesis arose from a preoccupation with the problem of nucleic acid biosynthesis. It is a curious fact that a phosphate diester linkage is restricted in its natural occurrence to nucleic acids and phospholipids, and we nurtured the vain hope that the elucidation of phospholipid biosynthesis would be more vulnerable to attack and thus provide a useful working model.

In view of the facility with which L-α-glycerophosphate

(α-GP) is formed in the body under both aerobic and anaerobic conditions, and the abundance of α-GP in various tissues, we guessed that it might prove to be a precursor in phospholipid synthesis. We were gratified to find that in the presence of liver extracts and certain activators there was a rapid and extensive formation of a phospholipid substance from α-GP (11). As shown in Table 2, the progress of reaction, as measured by the incorporation of P^{32} counts into an isolated phospholipid fraction, is dependent on the presence of ATP, Coenzyme A (*CoA*), and a long-chain fatty acid, and is abolished by brief heating of the enzyme fraction. I would like to point out that when this reaction was first observed, the experimental vessel contained 2 micromoles of α-GP (possessing 400,000 counts per minute) of which only about 100 counts per minute (equivalent to < .001 micromole) were incorporated in the phospholipid fraction. After optimal conditions for the reaction were worked out, the rate was raised several hundredfold and was many times faster referred to the weight of liver used, than the reported *in vivo* incorporation of P^{32} with phospholipids

TABLE 2

REQUIREMENTS FOR CONVERSION OF α-GP^{32} TO A PHOSPHOLIPID (11)

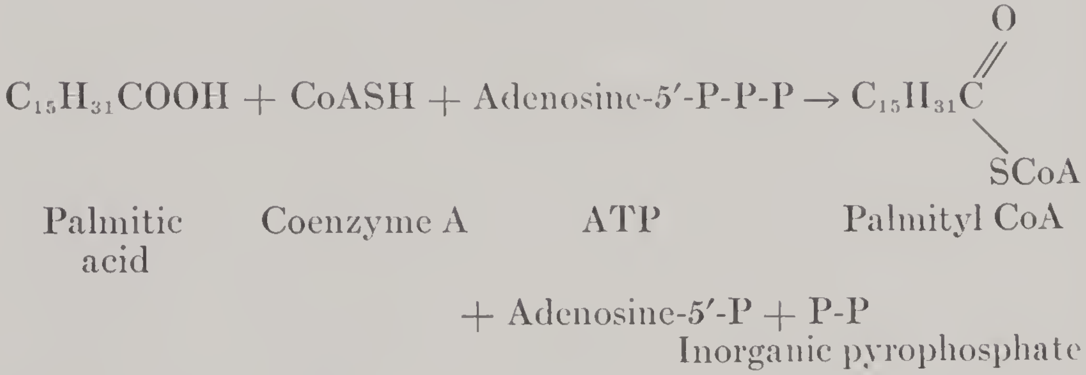
	Counts/min.*	μ moles **
Complete system	31,200	0.156
Without ATP	0	.000
Without CoA	0	.000
Without Fatty acid	2,210	.012
Enzyme heated 2 min. at 50°	0	.000
Inorganic P^{32} in place of α-GP^{32}	60	

* Radioactivity in the phospholipid fraction.

** μ Moles of α-GP esterified.

(9). Prepared by all that had been learned about acetate activation (12, 13), we were able to show that adenosine triphosphate (ATP) and *CoA* were required to activate the long-chain fatty acids according to Equation 4.

Equation 4



$$\underset{\text{Palmitic acid}}{C_{15}H_{31}COOH} + \underset{\text{Coenzyme A}}{CoASH} + \underset{\text{ATP}}{\text{Adenosine-5}'\text{-P-P-P}} \rightarrow \underset{\text{Palmityl CoA}}{C_{15}H_{31}C\overset{\displaystyle O}{\underset{\displaystyle SCoA}{}}} + \text{Adenosine-5}'\text{-P} + \underset{\text{Inorganic pyrophosphate}}{\text{P-P}}$$

The palmityl *CoA* was isolated and identified. Palmityl (or other long-chain fatty acid)-*CoA* was also synthesized by chemical methods. It was then shown that in the absence of ATP or free *CoA*, α-GP was esterified by palmityl-*CoA* (acyl *CoA*) to become a lipid substance. The phospholipid product was isolated and identified as a dipalmityl (diacyl) phosphatidic acid containing no choline, ethanolamine, serine or other nitrogenous compounds. At the same time that this work was done, Kennedy (14) independently made closely related observations on the conversion of inorganic P^{32} by liver mitachondria to lipid substances (very likely phosphatidic acids) and concluded that α-GP was an intermediate.

It was interesting to observe the influence of fatty acid chain length on the rates of activation of fatty acids and the rates of their esterification by α-GP. As shown in Figure 2, the ability to be converted to a *CoA* derivative was rather general, embracing odd- and even-numbered fatty acids of chain lengths from 5 carbons up. This finding is in keeping with what is now known about the need for activa-

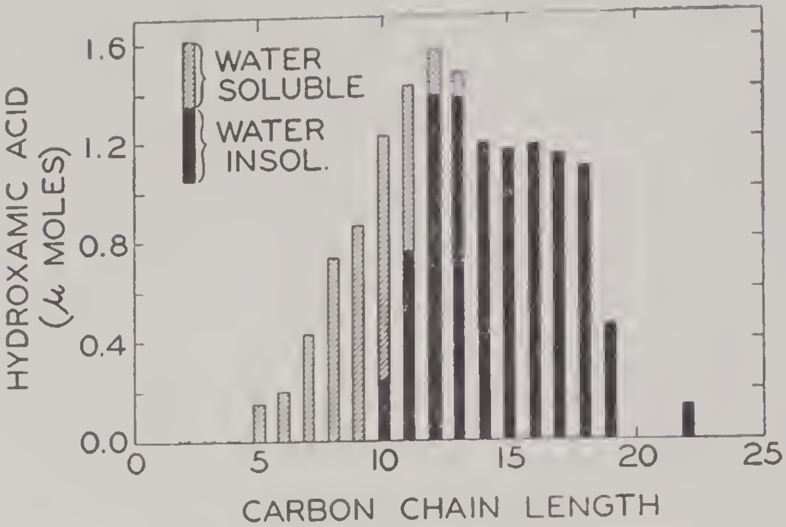


Figure 2. Influence of chain length of saturated fatty acids on rate of hydroxamic acid formation (11). The incubation mixture contained 0.29 ml. of ATP (0.05 M), 0.15 ml. of CoA (0.0638 M, 42 per cent pure), 0.15 ml. of cysteine (0.2 M), 0.05 ml. of $MgCl_2$ (0.3 M), 0.5 ml. of hydroxylamine (2 M solution of the hydrochloride adjusted to pH 7.4 with NH_4OH), 0.10 ml. of lyophilized residue fraction (39 mg. per ml.), 0.2 ml. of fatty acid (0.02 M), and water to a final volume of 2.0 ml. Incubation was for 30 minutes at 40°.

TABLE 3

INFLUENCE OF NATURE OF FATTY ACID ON RATE OF ESTERIFICATION OF α-GP (11)

Fatty acid	Activity	Fatty acid	Activity
Saturated		Saturated	
Lauric (C_{12})	2	Nonadecanoic (C_{19})	27
Tridecanoic (C_{13})	8	Arachidic (C_{20})	22
Myristic (C_{14})	12	Docosanoic (C_{22})	24
Pentadecanoic (C_{15})	34	Unsaturated	
Palmitic (C_{16})	67	Oleic (C_{18})	66
Margaric (C_{17})	100	Linoleic (C_{18})	26
Stearic (C_{18})	84	Linolenic (C_{18})	63
		Chaulmoogric (C_{18})	24

Fatty acid concentration 1×10^{-3} M. The esterification of 0.202 μM of α-GP^{32} obtained with margaric acid is assigned a value of 100 for ease of comparison with results obtained with the other fatty acids.

tion of all fatty acids in the course of their oxidative breakdown or synthesis. However, the effectiveness with which these various fatty acid-*CoA* derivatives esterify α-GP is quite different. As can be seen in Table 3, rapid rates of reaction occurred only with the C_{16}, C_{17} and C_{18} acids, an observation which may help to explain why tissue phospholipids rarely contain the fatty acids of shorter or longer chain length.

Now, finally, let us consider the biosynthesis of lecithin (Figure 3). The fact that phosphatidic acids are formed at very rapid rates by liver preparations, even when α-GP and acyl-*CoA* are present at very low concentrations, encourages us to think that phosphatidic acids are important intermediates in phospholipid synthesis. However, the nature of the mechanism involved is still a matter for speculation.

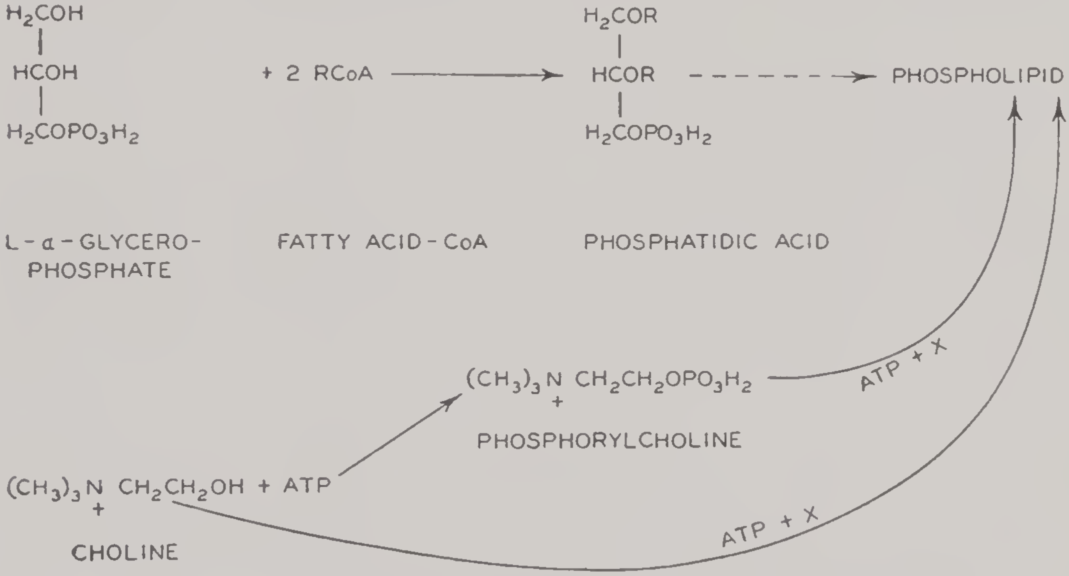


Figure 3. Hypothetical Pathways of Phospholipid Synthesis.

We (11) have observed that C^{14}-labeled phosphorylcholine is incorporated by liver extracts into an unidentified phospholipid. In fact, results with phosphorylcholine, doubly labeled with P^{32} and C^{14} appeared to indicate that it was incorporated as a unit. The observation that there is an enzyme in yeast and in most animal tissues which phosphorylates choline to phosphorylcholine (15) strengthens the view that phosphorylcholine may be a precursor. One of several possibilities is that phosphatidic acid may react with an enzyme to yield a diglyceryl-enzyme complex, which in turn reacts with phosphorylcholine or phosphorylethanolamine or phosphorylserine to form lecithin or cephalin. Kennedy (16) has recently shown that in liver mitachondria there is a pathway of phospholipid formation that utilizes choline rather than phosphorylcholine as a precursor. It is obvious that much more investigation is needed to clarify the workings in this interesting and important area of phospholipid metabolism.

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11. Some Aspects of Cholesterol Metabolism Related to Atherosclerosis

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CURRENTLY THE VIEW is widely held that atherosclerosis is primarily the result of a disordered cholesterol metabolism. Evidence supporting this concept has been derived from two principal lines of investigation. In the first place, it has been known for many years that cholesterol is present in the characteristic lesion of the disease, the atheroma. Quantitative measurements by Windaus, Schoenheimer (1, 2), and others (3, 4) have shown that cholesterol and its esters comprise the major lipid components of the atheromatous human aorta and of the atheromatous lesion itself. Secondly, it has been found that the administration of cholesterol to the rabbit, the chicken, the hamster, the guinea pig (5) and the thiouracil-treated dog (6) results in the production of arterial lesions which anatomically and chemically resemble those found in human atherosclerosis. In addition, the morphological stages in the development of the experimentally induced atheromatous process (7) parallel closely the sequence of anatomical events that is believed to occur in the development of the human lesion (8). The factual basis for the " cholesterol concept " of atherosclerosis has been comprehensively reviewed in an excellent recent monograph (5).

If then it be accepted as a working hypothesis that atherosclerosis is the result of a deranged cholesterol metabolism, it may properly be asked what is known concerning the nature of this aberration.

Chemical analyses of the lipids of the normal arterial intima, the atherosclerotic aorta, and of the early atheromatous plaque have revealed the presence in each instance of cholesterol, cholesterol esters, neutral fat, and phospholipid. Furthermore, in each case the ratios in which these lipids were present were very similar to their ratios in blood plasma but bore little relationship to their relative proportions in other tissues (1, 2, 3, 4). These findings have led to the concept that the cholesterol of the atheromatous lesion as well as that present in the normal arterial intima is derived from the blood plasma. More recently evidence has been obtained which indicates that aortic tissue itself is capable of synthesizing cholesterol from small molecules (9). However, the importance of this *in situ* formation as a factor in the pathogenesis of atherosclerosis has not yet been evaluated. Currently the plasma is regarded as at least the principal source of the lipids deposited in the atheromatous lesion.

Attempts to elucidate the factor or factors responsible for the deposition of plasma lipids in the vascular intima have been directed along two main channels. The first of these has been an endeavor to find a local abnormality in the arterial wall which would cause the deposition of lipids from a normal plasma. The second has centered about a search for defects or alterations in the plasma lipids which *per se* would would lead to atherosclerosis. Needless to say, some workers have suggested that a combination of these defects is necessary for the development of the disease process.

Although vascular injuries have been produced by a variety of experimental techniques (10, 11) no lesion closely resembling atherosclerosis has thus far been shown to occur as the result of arterial injury alone. However, it has been shown that areas of vascular trauma are sites of predilection for atheroma formation in animals which are subsequently rendered hypercholesterolemic by cholesterol feeding (11). Thus the hypothesis that atheromatosis may develop solely as the result of antecedent local changes has little convincing evidence to support it.

On the other hand, there is somewhat more evidence underlying the idea that some abnormality of the plasma is primarily responsible for the ensuing pathological changes in the arterial wall.

It has been reported that certain clinical entities such as hypothyroidism, nephrosis, and essential hypercholesterolemic xanthomatosis which are ordinarily accompanied by abnormally high plasma cholesterol concentrations are also accompanied by an increased incidence of atherosclerosis (5). Conversely, individuals having unusually low plasma cholesterol levels (around 160 mg. per cent) exhibit a marked reduction in the incidence and severity of atherosclerotic vascular disease (12). Although the status of hypercholesterolemia as an important etiological factor in the disease is still controversial, it seems probable that many, if not most, individuals developing clinically demonstrable atherosclerosis have plasma cholesterol concentrations which lie within accepted normal limits (13).

Because atherosclerosis appears to occur in the absence of hypercholesterolemia, numerous attempts have been made to demonstrate more subtle defects in the plasma, such as an altered phospholipid: cholesterol ratio (14), or defects in the lipid transport system not associated with abnormal lipid concentrations (15). Recently, the most prominent studies of this type have been carried out by Gofman and his associates (16). These workers have reported that normally most of the plasma lipids occur as soluble lipoprotein complexes rather than as the free compounds. Using the technique of ultracentrifugal flotation analysis, they have observed that a spectrum of lipoprotein molecules of varying size and density exists in human plasma. Furthermore, they have reported that although a low statistical correlation exists between the level of plasma cholesterol and the presence of coronary atherosclerosis, there is a much higher correlation between the disease process and elevated plasma concentrations of lipoprotein molecules having Sf values of 12–20. Moreover, it has been found that this group of lipoprotein molecules is absent from the blood of normal rabbits, but occurs in the blood of animals developing atherosclerosis as the result of cholesterol feeding (17).

As yet there is no general agreement that any of the above factors are etiologically related to human atherosclerosis. It would at least seem that there is no simple relationship between the plasma cholesterol concentration and the occurrence of the disease. However, evidence derived from comparative biology may be of interest in this connection. From studies of this type, several pertinent facts emerge. First, of the species thus far studied, man is the only mammal which is prone to develop atherosclerosis spontaneously (5). Next, among the mammals man has an average plasma cholesterol concentration which is uniquely high (18). Third, in those individuals having plasma cholesterol levels which approach those of the lower animals, the incidence and severity of atherosclerosis is very low (12). Fourth, atherosclerosis can be made to occur in the lower animals when their plasma cholesterol concentrations are artificially elevated by cholesterol feeding and related techniques. Although instances have been found in the lower animals in which hypercholesterolemia has occurred without inducing atherosclerosis (19), it has not been demonstrated that atherosclerosis occurs in these species in the absence of moderately or greatly elevated serum cholesterol levels. Finally, atherosclerosis of some degree has been reported to occur in almost 100 per cent of the adult population of North America (20). Even conservative estimates place the incidence of this disease at 50–90 per cent of the population past the age of 50 years (8). From these considerations, it might appear that the *average* plasma cholesterol concentrations found in man are not *normal* but pathologically high when viewed from the broader viewpoint of comparative biology. It may then be superfluous to argue whether a relatively small increment in an already elevated blood cholesterol will be significant in the production of atherosclerosis. It seems possible that beyond a threshold value, which may vary among individuals, the level of plasma cholesterol may only determine the rate at which lipids are deposited in the arterial wall.

If one accepts this concept as a working hypothesis, then the possibility exists that atherosclerosis could be prevented if it were feasible to lower the plasma cholesterol level to values now regarded as subnormal. In order to discuss possible means of accomplishing this, it will be necessary to consider some of the factors known to be operative in regulating the plasma cholesterol concentration.

Available evidence indicates that the plasma lipids are in a state of dynamic equilibrium (21), and that the concentration of cholesterol in the plasma at any time represents a balance between the rates of addition to the plasma and the rate at which the sterol is being removed from this system. These relations are depicted in Figure 1. In the absence of large quantities of dietary cholesterol, it appears that the liver is the primary source of cholesterol entering the system as well as the principal organ concerned in its removal (18). Although some of the peripheral tissues are capable of synthesizing and utilizing cholesterol (22), their contribution to the dynamic equilibrium of the plasma cholesterol is of minor quantitative importance under ordinary circumstances. The importance of the hepato-entero-vascular circulation of cholesterol in maintaining the plasma cholesterol level has not been assessed. However, investigation of this point is now being carried on in this laboratory.

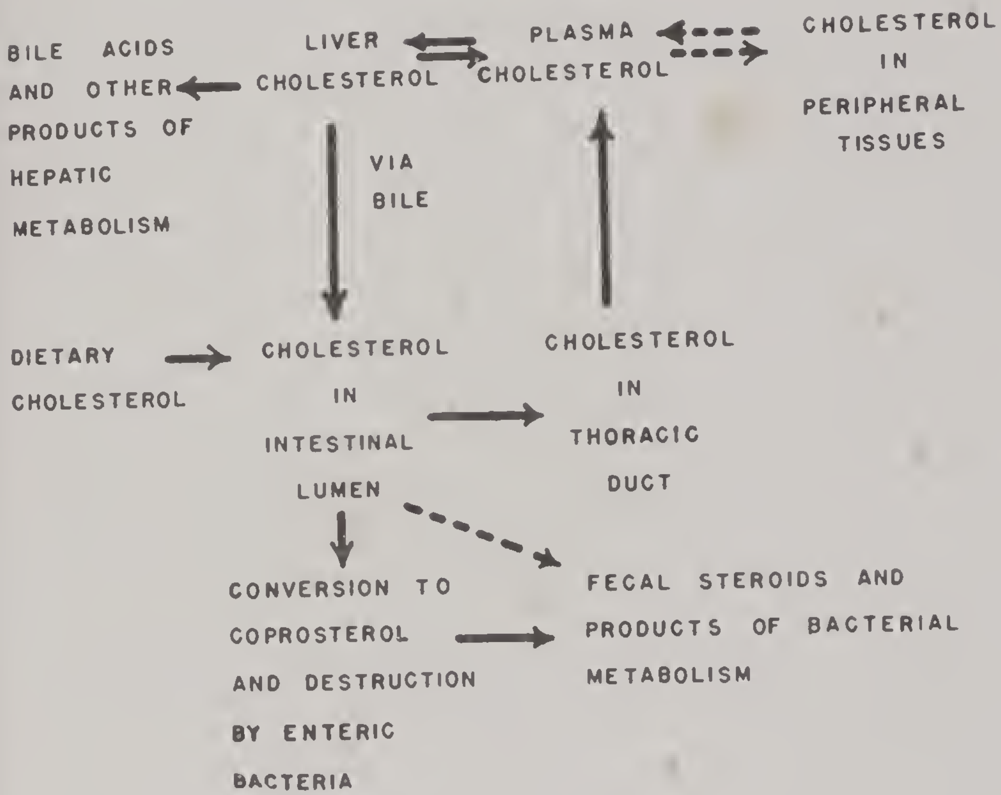

BILE ACIDS AND OTHER PRODUCTS OF HEPATIC METABOLISM
LIVER CHOLESTEROL
PLASMA CHOLESTEROL
CHOLESTEROL IN PERIPHERAL TISSUES
VIA BILE
DIETARY CHOLESTEROL
CHOLESTEROL IN INTESTINAL LUMEN
CHOLESTEROL IN THORACIC DUCT
CONVERSION TO COPROSTEROL AND DESTRUCTION BY ENTERIC BACTERIA
FECAL STEROIDS AND PRODUCTS OF BACTERIAL METABOLISM

Figure 1. Some interrelationships of the cholesterol of blood plasma and other systems.

From a consideration of the diagram it would seem that a decreased plasma cholesterol concentration might result from either a decreased rate of hepatic cholesterol synthesis, an increased rate of excretion of cholesterol via the gut, or a combination of these. Of course, other possibilities exist, but will not be discussed here. In the following sections, information bearing on the first two possibilities will be presented in greater detail.

"Cholesterol Biosynthesis," Intermediary Metabolites

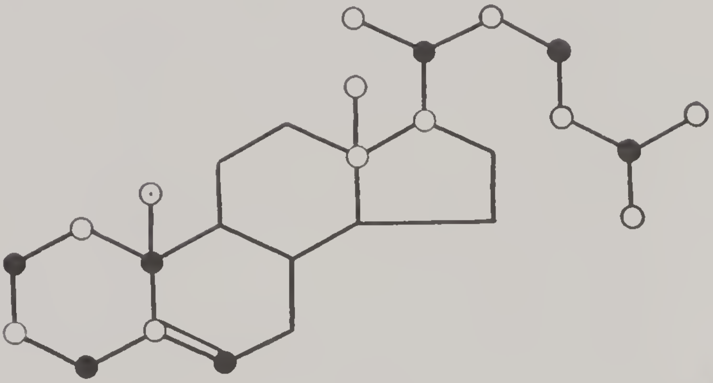
Although animal diets ordinarily contain varying quantities of cholesterol, it has been known for years that this compound is not an essential dietary constituent, and that animals are capable of synthesizing the sterol. Conclusive evidence for this endogenous synthesis as well as clues to the nature of the molecular building blocks utilized were provided by the now classical experiments of Rittenberg and Schoenheimer (23). These workers found that deuterium enrichment of the body water of rodents resulted in an incorporation of the isotope into the tissue cholesterol of these animals. Since it was found that almost one-half of the hydrogen atoms of the sterol molecule had their origin in the body water, these investigators concluded that the biological synthesis of cholesterol involved the condensation of a large number of small molecules. The rate of sterol synthesis could also be obtained from their data, and it was found that, contrary to previous concepts, tissue cholesterol was being synthesized and degraded at a fairly rapid rate. It has been calculated (18) from more recent data that the average human adult synthesizes approximately 2 gm. of cholesterol daily. Since the pioneer work of Schoenheimer and Rittenberg, a rather large body of evidence has been obtained pertaining to the molecular precursors of cholesterol, the tissues active in the synthetic process, and some factors affecting the rate of synthesis.

At present it is thought that in the initial step in sterol synthesis, two molecules of acetic acid in the metabolically active form of acetyl coenzyme A condense to yield acetoacetyl coenzyme A. Evidence for the ability of acetic acid to serve as a cholesterol precursor is clearcut and unequivocal. Isotopically labeled acetate is converted to cholesterol by animal tissues both *in vivo* (24) and *in vitro* (25, 26). In a Neurospora mutant rendered genetically incapable of producing acetate from carbohydrate, it has been found that exogenous acetate can serve as the sole carbon source in the synthesis of ergosterol (27), the characteristic sterol of this mold. Moreover, cholesterol, obtained by biosynthesis from isotopic acetic acid, has been partially degraded and the carbon atoms derived from individual positions of the molecule have been isolated (28, 29, 30). These studies have shown that each position of the steroid molecule contains carbon originating specifically in either the carboxyl or the methyl group of acetate (Figure 2). Recently Lipmann and coworkers have obtained evidence which is indicative of the participation of coenzyme A in steroid synthesis (31).

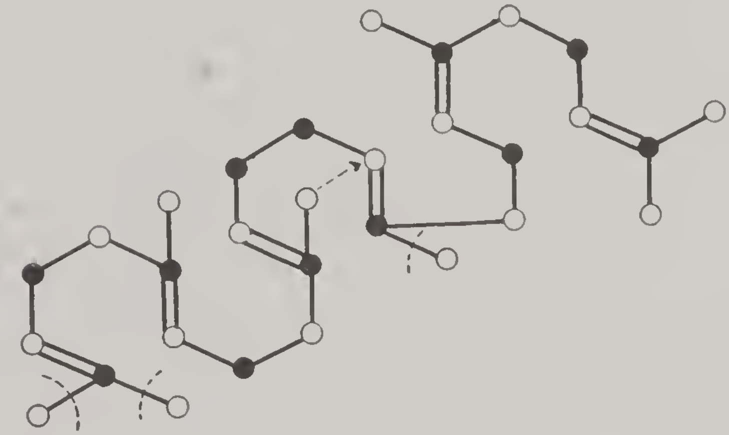
Although acetoacetic acid has been proposed as an intermediate in sterol synthesis, whether or not this compound lies on the direct route leading from acetate to cholesterol is an open question. The efficiency of labeled acetoacetate as a precursor of cholesterol in surviving liver tissue is somewhat lower than that of acetate, and prior breakdown to the two carbon acid followed by recondensation could easily account for the extent of conversion which has been observed in the liver slice (32, 33). There is more indirect evidence which also suggests that acetoacetate is involved in cholesterol synthesis (34, 35). However, until more convincing data have been obtained, the participation of this keto acid in sterol synthesis must remain in doubt.

The next compound that can be assumed with reasonable confidence to occupy the place of an intermediate in sterol synthesis is the hydrocarbon squalene. Since the original isolation of this compound from shark liver oil, it has been found to occur in a variety of animal and plant tissues. Shortly after the structure of squalene had been elucidated

(36), it was suggested that the hydrocarbon might be a sterol precursor. Balance studies designed to test this hypothesis yielded conflicting results (37, 38). Recently, however, more direct evidence has been obtained on this score by the application of isotopic tracer methods (39, 40, 41, 42). It has been found that labeled acetic acid is converted to squalene by



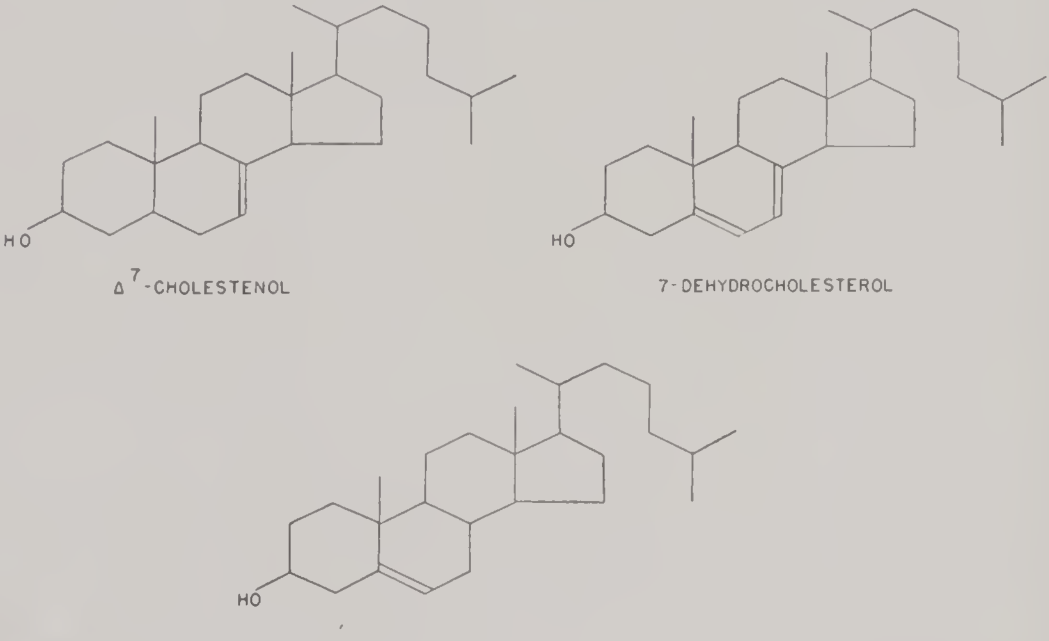
CHOLESTEROL



SQUALENE

Figure 2. The upper structure depicts the distribution of acetate carbon atoms found experimentally in cholesterol. The lower figure illustrates the hypothetical distribution of acetate carbon atoms in squalene and a possible mechanism of squalene cyclization (29). ● and ○ denote carbon atoms originating in the carboxyl and methyl groups of acetate respectively.

mammalian liver, both *in vivo* and *in vitro*. In this biosynthetic process, both the carboxyl and methyl carbons are utilized. With the aid of biosynthetic C_{14}-squalene it has been possible to show that intact animals convert the hydrocarbon to cholesterol with such efficiency and specificity that a direct cyclization of the squalene molecule (Figure 2) to the polycyclic steroid structure is rendered highly probable. The possibility that this conversion occurs via a breakdown to acetic acid followed by a recondensation to the sterol is rendered improbable by two facts. First, labeled squalene is converted to cholesterol fifty times as efficiently as labeled acetate. Secondly, there is virtually no conversion of squalene to fatty acids, while acetic acid serves as an efficient carbon source in their biosynthesis. The argument for a direct cyclization of squalene is further strengthened by a consideration of certain contributory evidence. The experimentally determined distribution of the carboxyl and methyl carbons of acetic acid in the cholesterol molecule is depicted in the upper structure of Figure 2. It may be noted that the terminal 5-carbon unit of the cholesterol side chain can be regarded as a hydrogenated isoprene unit. If it is assumed that each of the six isoprene units of which squalene is composed has the same distribution of acetate carbon atoms as does the terminal isopentyl unit of the cholesterol side chain, then the cyclization scheme in the lower part of Figure 2 would yield a polycyclic structure embodying both the structure of cholesterol and the acetate distribution found experimentally in the sterol. Further evidence for the participation of squalene as an intermediate in cholesterol synthesis has been obtained in the following manner. When cholesterol is administered to an animal, the hepatic cholesterol concentration increases and concomitantly the rate at which the liver converts labeled acetate to cholesterol diminishes markedly. This decreased rate of synthesis can be demonstrated to occur in the intact animal (43), the surviving liver slice (44), and in cell-free liver preparations (45). Since this effect is not accompanied by a decreased rate of fatty acid synthesis, a generalized interference with acetate utilization is excluded. When squalene rather than cholesterol is administered, the hepatic concentrations of both squalene and cholesterol increase, and the rate of sterol synthesis is depressed with the same specificity and to the same extent as it is after the feeding of cholesterol itself (42). In addition (46), the ingestion of squalene results in the accumulation in the liver of Δ^7-cholestenol, (Figure 3) a steroid which has been found by Fieser to be a congener of cholesterol in animal tissues (47).



Δ^7-CHOLESTENOL

7-DEHYDROCHOLESTEROL

CHOLESTEROL

Figure 3. The structures of cholesterol, Δ^7-cholestenol, and 7-dehydrocholesterol.

The administration of Δ^7-cholestenol to animals is followed by an increased hepatic sterol concentration and a decreased rate of sterol synthesis by the liver (48). The sterol which accumulates has been found to be cholesterol rather than the administered compound itself. The combined observations that Δ^7-cholestenol accumulates after squalene feeding, and is itself apparently converted to cholesterol suggest that this sterol is an intermediate in the conversion of squalene to cholesterol.

Another sterol which may enjoy intermediary status in the squalene-cholesterol conversion is 7-dehydrocholesterol (Figure 3), i. e., provitamin D3. In feeding experiments (48) this steroid has been found to behave in a manner very similar to that found for cholesterol and Δ^7-cholestenol. However, this compound has not been found to accumulate after squalene feeding.

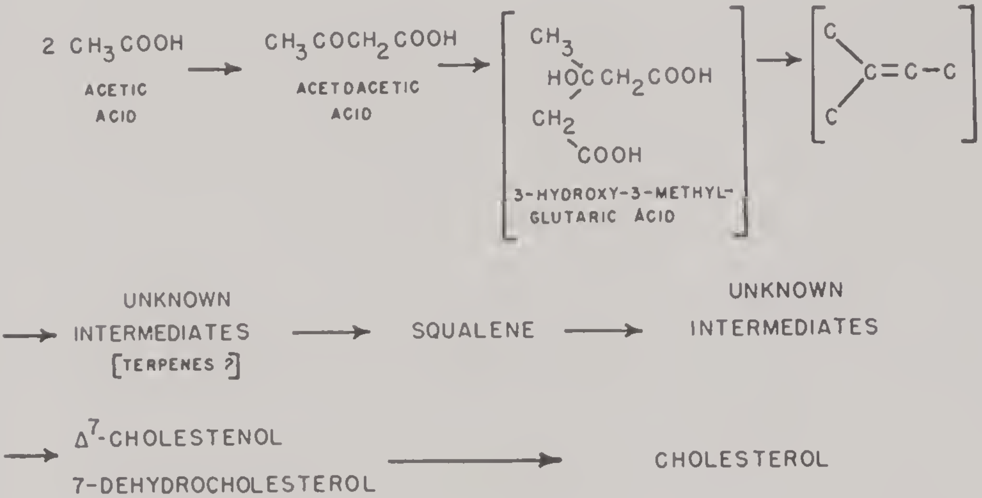


Figure 4. Possible pathway of cholesterol synthesis. Entirely hypothetical intermediates are enclosed in brackets.

Present ideas about the nature of the intermediary compounds involved in cholesterol synthesis are summarized in Figure 4. It is obvious that this series is largely hypothetical and is certainly far from complete. However, the experimental groundwork has been laid upon which future research can be reasonably based.

At this point it may be noted that the hydrocarbon squalene is but one member of the extremely large group of naturally occurring susbtances known as terpenes, which are characteristically composed of two or more isoprene units. A few other examples of this type of compound are rubber, vitamin A, camphor, and phytol, a constituent of the chlorophyll molecule. Since a terpene has now been found to be an intermediate in cholesterol biosynthesis, it may become feasible to apply closely the results gained from investigations of terpene synthesis in plants and microorganisms to the problems of steroid synthesis.

With the advent of further knowledge it is possible that competitive inhibitors of cholesterol synthesis can be prepared, and that the rate of endogenous sterol formation can be controlled at will.

"Cholesterol Biosynthesis," Factors Affecting the Rate of Hepatic Synthesis

In the preceding section it was pointed out that the dietary administration of certain sterols led specifically to a decreased rate of cholesterol synthesis by the liver and an increased sterol concentration in that organ. The mechanism of the decreased synthetic capacity under these circumstances becomes of interest when it is considered that a particularly vulnerable link in the chain of reactions leading to cholesterol may be involved. Since the synthesis of cholesterol appears to be an irreversible process in that totally different pathways are followed in the synthesis and degradation of the compound, it appears unlikely that a decreased synthetic rate would result from a mass action effect. However, the actual mechanism is obscure.

Caloric restriction is also known to decrease the rate of hepatic cholesterol synthesis. In the rat, deprivation of food for only twenty-four hours depresses the rates of sterol and fatty acid synthesis by isolated hepatic tissue to about one-tenth the rate observed in liver slices from well fed animals. Fasting for thirty-six hours produces a more profound depression (49). However, if prior to sacrifice the fasted animal is refed with carbohydrate, protein, or fat, its hepatic tissue synthesizes cholesterol two to ten times as rapidly as does tissue from animals which have been maintained on a normal diet (49). Little is known about the mechanism of these caloric effects.

Essential dietary constituents have also been shown to have effects on sterol formation. It has been reported that the livers of scorbutic guinea pigs synthesize cholesterol at a rate about fifty per cent greater than control animals (50). On the other hand, the livers of rats deficient in pantothenic acid have been found to show a decreased capacity for sterol synthesis (31). Although the role of ascorbic acid is unknown, the effect of pantothenic deficiency is most probably related to the fact that this vitamin is an integral part of the coenzyme A molecule.

The rate of cholesterol synthesis has also been found to be subject to various hormonal effects. Extirpation of the hypophysis lowers the rate of hepatic sterol synthesis by a factor of one hundred in the rat (51). In the same species, hypothyroidism decreases both the rate of synthesis and the rate of excretion, while hyperthyroidism induces exactly the opposite effects (52). Recently it has been reported that the liver of the alloxan diabetic rat manufactures cholesterol at an increased rate (53). Although possible explanations have been advanced to explain these hormonal effects, in no case is the mechanism of action actually known with any degree of certainty.

The Biliary Excretion and Intestinal Absorption of Cholesterol

Since limitations of space preclude a more comprehensive discussion of the metabolism of cholesterol in the gut, only a few of the many interesting aspects of this problem can be considered here.

Human bile normally contains substantial quantities of cholesterol which is entirely in the free (unesterified) form. It has been estimated that 0.5–1.0 grams of cholesterol enters the human gastrointestinal tract by this route daily. Since this quantity is equivalent to 25–50 per cent of the cholesterol produced by endogenous synthesis daily (18), the question of its further fate becomes of interest.

For many years it has been known that the presence of neutral fat in the diet greatly facilitates the absorption of dietary cholesterol from the gut. More recently it has been found that the presence of a bile salt is absolutely essential for the absorption of carbon-14 labeled cholesterol from the intestinal tract (54).

It is also known from tracer studies that over 90 per cent of the cholesterol absorbed from the intestinal tract is transported to the blood via the intestinal lymphatics and the thoracic duct. Moreover, although the cholesterol of both the diet and the bile is entirely in the free form, at least 50 per cent of the absorbed cholesterol is found to be present as cholesterol esters in the thoracic duct lymph (55).

This esterification may be accomplished through the action of cholesterol esterase, an enzyme secreted by the pancreas which catalyses the reversible hydrolytic cleavage of cholesterol esters. Interestingly enough this enzyme requires for activity the presence of cholesterol, a source of fatty acids, and a bile salt, the same trio which has been shown to be essential for the intestinal absorption of cholesterol. In addition, certain structural analogues of cholesterol have been found to inhibit both cholesterol esterase activity and the intestinal absorption of cholesterol (56). These observations suggest that the esterification of cholesterol, catalyzed by cholesterol esterase, is an essential feature in the absorption of the sterol from the gastrointestinal tract. Several interesting questions are raised by this possibility. It would be of interest to know if the enteric esterification of cholesterol is of major importance in maintaining the relatively constant ratio of free cholesterol to cholesterol ester found normally in serum. If such were the case, it would help to explain the high free cholesterol: cholesterol ester ratio found in obstructive jaundice. Perhaps of greater interest is the possibility that the rate of biliary cholesterol excretion and the extent of intestinal reabsorption of the sterol from this source are of importance in regulating the concentration of cholesterol in the serum. In this connection may be mentioned the observations by Keys (57), and others, that a diet low in neutral fat is much more effective in lowering serum cholesterol levels than is a diet low in cholesterol itself. Since neutral fat functions both as a chologogue and as a source of fatty acids, it might be expected that a high fat diet would lead to the reabsorption of endogenous cholesterol which would otherwise be excreted in the feces (Figure 1). A much more detailed review of the enteric metabolism of cholesterol may be found in reference (12).

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